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**Characterising the functional role of
rhizosphere fungi in *Miscanthus giganteus*
bioenergy cropping systems**

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Declaration

I declare the work presented in this thesis was conducted by me, under the direct supervision of Dr. Gary D. Bending, Dr. Niall McNamara, and Dr. Chris van der Gast, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the work here has been previously submitted for any other degree.

Abstract

The rhizosphere has a rich fungal microbiome, including parasites, commensals and mutualists. An important group in the rhizosphere are assumed to be the arbuscular mycorrhizal fungi (AMF), which live in symbiosis with around 80% of plant species. AMF have been shown to increase plant yield, biomass, disease resistance, and shoot P. Plants exchange carbon in the form of sugars for nutrients assimilated by AMF. There is little known about AMF in association with *Miscanthus giganteus*, a productive bioenergy crop grown in the UK and abroad. Work was carried out to characterise the abundance, organisation, importance, function and stability over space and time of rhizosphere fungi and AMF in *M. giganteus* roots. Field samples from Lincolnshire were analysed using staining and molecular techniques, including small subunit rRNA gene terminal restriction fragment length polymorphism, clone libraries and amplicon pyrosequencing, and meta-transcriptomics. *M. giganteus* was also grown in a number of pot experiments, with various treatments including fungal inoculations and fungicide application. A number of fungal phyla were found in the roots, particularly Ascomycota, the composition of which shifted over time and exhibited diurnal patterns of activity. Fungi enhanced plant growth by a third, and were functionally active in the roots in the meta-transcriptome. AMF communities were found at much lower relative abundances in roots, and inoculation with AMF did not enhance *M. giganteus* growth. The work highlights the importance of the whole root mycobiome to plant growth and health, and the relatively small role Glomeromycota play in *M. giganteus* comparison with other fungi. The work also demonstrated the dynamic nature of fungal activity over hours, months, and years, and the complex interactions the fungal community has with environmental variables.

Chapter 1. Introduction

1.1 The rhizosphere

The rhizosphere is the area surrounding plant roots, comprising soil, microbes, and molecules, which are directly affected by water and nutrient uptake, and rhizodeposits, such as sloughed off root cells, proteins, sugars, and hormones, all of which attract and sustain plant-microbe interactions belowground (Berendsen, *et al.*, 2012). Microbes are extremely diverse and abundant in soil. It is estimated that in one gram of soil there are 10^{10} - 10^{11} bacterial cells (Horner-Devine, *et al.*, 2003). However, in the majority of literature, fungi are found to be the most dominant microbial group, with a mean varying between 35 - 76 % of the total microbial biomass, in different soil types (Joergensen and Wichern, 2008). The distinctive chemical and physical environment of the rhizosphere drives the selective growth of microbes, particularly those showing fast growth and chemotaxis (Hartmann, *et al.*, 2009). Despite this selection, there is an incredible abundance of microbial cells in the rhizosphere, including typically 10^{11} bacterial cells in one gram of root (Egamberdieva, *et al.*, 2008). The rhizosphere biota has been referred to as the second genome of the plant because of the intimate nature of the interaction between it and the plant (Berendsen, *et al.*, 2012).

Life in the rhizosphere is highly dynamic, and intimately linked to plant productivity and nutrient cycling. Rhizosphere microbes can be directly beneficial to plant growth, particularly symbionts such as nitrogen fixing bacteria and mycorrhizal fungi (Kaschuk, *et al.*, 2010). Symbionts enhance plant uptake of a range of nutrients, particularly P and N, and may play a role in water uptake and inducing disease resistance (Morgan, *et al.*, 2005). Additionally, free living soil microbes can be indirectly beneficial to plants, by stimulating nutrient availability and suppressing pathogens. For example, saprophytic bacteria and fungi break down up to 100 % of leaf litter, and in doing so release nutrients into the environment for plants and other microbes to acquire (Hattenschwiler, *et al.*, 2005). Microbes also synthesise binding agents, which improve soil aggregation, leading to structural changes which can benefit root growth (Six, *et al.*, 2006). For instance arbuscular mycorrhizal fungi have been reported to

produce glomalin, a long lasting protein which improves aggregate water stability and soil structure (Rillig, 2004; Rosier, *et al.*, 2006). It supports bacteria which produce anti-microbial agents, for example *Streptomyces* extensive catalogue of antibiotics (~3,000) (Clardy, *et al.*, 2006), and fungi which produce anti-fungal metabolites, such as extracellular chitinases, which trigger defense mechanisms in the plant to enhance resistance to pathogens. These interactions are responsible for suppressive interactions, which can be promoted by some soil management practices, which improve microbial diversity, and have also been used to develop specific biocontrol agents (Whipps, 2001). In general, it is thought a higher diversity of microbes is more beneficial to soil and plant health, to fill the many niches of nutrient cycling, symbiotic plant-microbe interactions, and plant disease suppression.

However, other microbial processes can have negative impacts on plant growth. For example, microbes can compete directly with plants for growth limiting nutrients such as N (Jackson, *et al.*, 1989), reducing availability to plants; denitrification results in loss of N to the atmosphere (Vitousek and Howarth, 1991). The rhizosphere is also home to many pathogens, which can specifically or opportunistically parasitise plants (Whipps, 2001).

1.2 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF), in the phylum Glomeromycota, form interactions with ~80% of herbaceous land plants, and evolved ~460 million years ago (Smith and Read, 2008). Currently, 241 AMF species have been described morphologically, and around 341 with molecular methods (Opik, *et al.*, 2013; Schüßler and Walker, 2014). The word mycorrhiza is derived from the Greek words 'mykes' meaning fungus, and the latin word 'rhiza' meaning root, and arbuscule is derived from the latin word 'arbusculum' meaning small tree, in reference to the morphological structure of arbuscules in the roots. AMF form arbuscules in root cortex cells, to exchange fungal nutrients for photosynthetic sugars (Parniske, 2008). It is estimated that globally, 5 billion tons of carbon flows into AMF through plants per year (Bago, *et al.*, 2000), so they are potentially very important components of the terrestrial C cycle.

1.2.1 Importance of AMF for plant nutrition

AMF are obligate symbionts, and have been shown to increase plant yield, biomass, and shoot P concentration (Lekberg and Koide, 2005). In exchange, plants provide AMF with what is believed to be their sole carbon source, which can amount up to 20 % of newly fixed plant carbon (Bago, *et al.*, 2000; Vandenkoornhuyse, *et al.*, 2007). AMF are known to expand the horizon of the root system, which can improve nutrient and water uptake. In addition, it is generally accepted that nutrient exchange between AMF and plants is an essential component of the mutualistic biotrophy, but the level of exchange and benefit to the plant can vary widely depending on the host, fungal species, experimental conditions, soil type, and level of fertilization (Smith and Read, 2008).

A meta-analysis of 290 published trials showed that AMF inoculation overall improved yield by 23 %, biomass by 57 %, and shoot P by 33 %, while shortened fallow enhanced AMF colonisation and increased plant biomass by 55 % (Lekberg and Koide, 2005). However, increased colonisation reduced productivity in 2 % of trials, and was less beneficial when soil P was high (Lekberg and Koide, 2005). Other trials have shown neutral or negative growth responses to AMF inoculation (Larimer, *et al.*, 2010; Li, *et al.*, 2008), showing that AMF are not always beneficial. Another meta-analysis of 134 publications looked at the net effect of various factors on plant benefits from AMF. C₄ grasses and non-N-fixing plants responded better to AMF inoculation than C₃ grasses and N-fixing plants (Hoeksema, *et al.*, 2010). AMF inoculation was also more effective with higher soil microbial diversity, and when soil was P-limited rather than N-limited (Hoeksema, *et al.*, 2010).

The most widely studied, and thought to be most important, nutritional exchange is between plant C and fungal P. Forms of P in soil include inorganic-P, which can be taken up by plants and microbes, and organic P, which is unavailable for plant uptake (Smith and Read, 2008). Plants and fungi have a number of strategies for inorganic P uptake, including release of organic acids to compete with chelates, topsoil foraging and increasing surface area for more uptake (Xiao, *et al.*, 2005). However, hyphae are up to 100 times longer than root hairs, and are therefore more efficient foragers. AM can also stimulate secretion of plant derived phosphatases (Ezawa, *et al.*, 2005).

The AM route for plant P uptake can be substantial. Grace et al (2009) showed that 48% total plant P originated via the AM pathway in *Hordeum vulgare* in symbiosis with *Glomus intraradices*. The fungal route begins when P is taken up by AM mycelia, and is then transported along hyphae as poly-P, before solute C and P are actively exchanged in arbuscules across the periarbuscular membrane (Harrison, et al., 2002). There is also evidence that AMF have a role in plant N acquisition. N is a limiting resource, and plants mainly rely on saprotrophic microbes to release inorganic NH_4^+ from organic-N, during decomposition of organic materials. AMF have been shown to uptake and transport NH_4^+ (Ames, et al., 1983; Govindarajulu, et al., 2005), but also to accelerate decomposition of leaf litter, then translocate assimilated N to plants (Hodge, et al., 2001). While P and N are important macronutrients, AMF have been shown to supply a range of nutrients to plants in experimental chambers, including, SO_4^{2-} (Casieri, et al., 2012), Cu, K, Ca, Zn, and Fe (Birhane, et al., 2012; Marschner and Dell, 1994).

1.2.2 Other roles of AMF

AMF have a number of other functions, including reports that AMF produce the glycoprotein glomalin, which improves soil stability and adhesion (Wright and Upadhyaya, 1998), amino acid uptake (Roberts and Jones, 2012), and can regulate water use efficiency in plants (Birhane, et al., 2012). Single AMF strains can colonise multiple hosts, and their external mycelia can form a 'common mycelial network', which allows the transfer of molecules, including carbon and nutrients, between host plants (Simard, et al., 2002; Smith and Read, 2008). Through the common mycelial network, AMF can protect plants against disease. For example, AMF transfer signals warning neighbouring plants of aphid or pathogen attack, and use allelochemicals to regulate plant community structure (Babikova, et al., 2014; Jung, et al., 2012; Newsham, et al., 1995; Song, et al., 2010).

To attract mycorrhizal fungi, plants release hormones called strigalactones, and in response AMF release 'Myc factors', similar to 'NOD factors' in legume symbiosis, which are lipochitooligosaccharide diffusible symbiotic signals. Myc factors have been detected in a wide range of host plants, and have been shown to stimulate root growth and branching, and a symbiotic signal transduction pathway in *Medicago trunculata* (Maillet, et al., 2011). In addition,

Glomus intraradices was recently found to secrete small secreted proteins to suppress the host immune systems for establishment (Kloppholz, *et al.*, 2011). The discovery of strigalactones and Myc factors suggests that both partners seek the other one out, as part of a complex signaling pathway, to initiate mutualistic relations.

1.2.3 Factors affecting AMF colonisation

AMF are found in most herbaceous species, but the factors affecting their abundance and colonisation of roots are still not fully understood. AMF diversity and abundance may be influenced by various environmental factors including soil pH, moisture, nutrient availability, and plant diversity (Hartnett and Wilson, 1999; Torrecillas, *et al.*, 2012; van der Heijden, *et al.*, 2008; van der Heijden, *et al.*, 1998). The addition of P fertiliser was shown to consistently reduce the colonisation of roots by AMF by an average of 32% across twenty field studies (Treseder, 2004), and decreased diversity (Gosling, *et al.*, 2013). If nutrient levels are satisfied, plants down-regulate carbon flow to AMF (Bauen, *et al.*, 2010). In addition, organic farming methods have been shown to have higher AMF abundance, compared to conventional practices (Gosling, *et al.*, 2006). The lower diversity of AMF in conventional agricultural systems is thought to be linked to practices such as tillage, biocides, monocultures and crop rotation, which all destabilise microbial communities, and the use of water-soluble fertilisers, including N (Leake, *et al.*, 2004), which release nutrients quickly into the soil thereby negating the plant's need for symbiotic nutrient exchange (Bainard, *et al.*, 2012; Gosling, *et al.*, 2006; Schalamuk, *et al.*, 2011). In general, it is believed that agricultural soils have a lower diversity and abundance of AMF compared to natural systems. However, the diversity of AMF in *Trifolium repens* across habitats in Ireland was found to be influenced by abiotic factors (rainfall, pH and soil type), but not land use (Hazard, *et al.*, 2013).

1.2.4 AMF genome

The 153 Mb genome of *Glomus irregularis* has been sequenced and annotated, with very interesting findings about the lifestyle of AMF (Tisserant, *et al.*, 2013). There was an unusually high number of genes involved with phosphorous metabolism and phosphorylation signaling, nitrate transporters, and sulfite reductases, in *G. irregularis*, compared with similar sized fungal genomes. The

inflated abundance of nutrient transporters supports previous evidence that AMF enhance plant nutrition. In the genome there was an absence of a number of functional genes encoding enzymatic degradation of plant cell wall components such as, lignin, polysaccharides, cellulose, and cellobiose, and the production of secondary metabolite toxins. The genomic downsize is thought to be an evolutionary adaptation to the obligate lifestyle, which reduces the plant host response to effector molecules, and allows AMF entry into the root cortex. There was also an absence of thiamine biosynthesis, and sucrose transporters. Together with the lack of plant cell wall degrading enzymes, this suggests that AMF rely on plant enzymes and transporters for the development of arbusculated cells, and to supply their sole source of carbon and vitamin-B (Tisserant, *et al.*, 2013). The genome provides a wealth of information, and a resource to annotate Glomeromycota genome and transcriptome data.

1.3 Techniques to characterise fungal diversity and function

Traditionally fungi and AMF have been characterized using spore, hyphal, and arbuscule morphology (AMF only). More recently, molecular techniques, including Sanger sequencing of small subunit (SSU) rRNA gene clone libraries, and terminal restriction fragment length polymorphisms (TRFLP) of SSU rRNA genes, have been used to estimate diversity, but it is difficult to assign the phylogeny of TRFs accurately, and clone libraries only consist of small sample sizes, typically of ~90 clones. The latest technology to characterise fungal diversity is the high throughput sequencing of taxonomically informative genomic regions, mainly the ITS region and SSU rRNA gene, using Roche 454 or Illumina equipment, which provide taxonomic and frequency data of thousands to millions of sequences with ever decreasing costs (as little as £5 per sample).

Currently (2014), research is shifting to a more functional approach, to detect active genes within communities, such as those contributing to P transport (Gamper, *et al.*, 2010). Functional approaches have been well used to characterise microbial communities involved in various soil processes, including nitrogen cycling and pollutant degradation (Hollister, *et al.*, 2010; McDonald, *et al.*, 2010). Technology is now available to sequence whole genomes, which is becoming faster, cheaper, and providing increasingly large sequence coverage over time. Furthermore the inclusion of functional genes, and annotation of

genomes, within online databases, has enabled the annotation of many more plant and fungal transcriptional profiles. Microarrays have been used to monitor transcriptional changes in the development of potato in symbiosis with AMF, identifying a suite of specific plant genes involved in the symbiosis including some associated with stress, development, cell wall construction and transport (Gallou, *et al.*, 2012).

Meta-genomics is the concept of sequencing all genomic material in a microbial community, at the same time, which provides an insight into the structure and function of a community. For example, meta-genomics has been used to catalogue the genes of human gut microbes (Qin, *et al.*, 2010). However, the starting material for meta-genomics is DNA, therefore it can only provide information on the presence of genes, which are only potentially expressed. A meta-transcriptome, on the other hand, is the simultaneous characterisation of structure and function of the active community, and can therefore be used to differentiate the ecological roles of microbial groups. There are a number of different starting materials possible for meta-transcriptomics, but total RNA and messenger RNA (mRNA) are most commonly used. Total RNA consists of > 80 % of ribosomal RNA (rRNA) (Palmer and Prediger 2014), and therefore a meta-transcriptome with total RNA will overwhelmingly characterise taxonomy of active communities, and very little functional activity will be detected, which was the case for Turner, *et al.* (2013). However, selecting and enriching mRNA, which represents a much smaller proportion (~ 1 - 3 %) of total RNA (Palmer and Prediger 2014), for a meta-transcriptome, allows the assessment of the transcriptional activity of communities, which includes functional genes, transcription factors, and taxonomic information (Damon, *et al.*, 2012). Eukaryote mRNA can be separated from prokaryote and non-coding RNA by poly-A tail extraction (Grant, *et al.*, 2006), which further improves the proportion of transcriptomic information sequenced, for fungi and other eukaryotes. A meta-transcriptome of eukaryote gene expression in forest soil has recently been published, which described a range of genes, including microbial mRNA involved in nutrient acquisition, metabolism, cell wall and chitin degrading enzymes and membrane transport proteins, in addition to phylogenetic markers (Damon, *et al.*, 2012). Next generation sequencing techniques will help us to understand the function and ecology of microbes in different environments, on a scale not possible with traditional culture-dependent and morphological based studies.

1.4 *Miscanthus x giganteus*

Miscanthus x giganteus, from now on referred to as *M. giganteus*, is a rhizomatous perennial grass, native to the tropics and subtropics of Asia and Africa. It is now grown as a biomass crop, to create energy from combustion or bio-based products, and covers over 15,000 ha in the UK (NNFCC, 2008). *Miscanthus sp.* performs C4 photosynthesis, which has a higher efficiency of carbon fixation, nutrient and water use, than C3 plants. *M. giganteus* is a sterile hybrid which is propagated from rhizomes. Rhizomes are storage organs which sequester assimilates for rapid shoot growth in the next growing season (Suzuki and Stuefer, 1999). *M. giganteus* has been shown to input more carbon into the soil than other agricultural and bioenergy crops, which is thought to be due to its productive, C4, rhizomatous growth (Hansen, *et al.*, 2004; Khale, *et al.*, 2001). For example, fatty acids in the rhizosphere of a German biomass cropping system were 35 - 70 % derived from *M. giganteus* (Weisenberg, *et al.*, 2012). *M. giganteus* is very water efficient, even though a high percentage of rainfall is intercepted by leaves (Finch and Riche, 2010). *M. giganteus* is cold tolerant, growing at 6°C, and has a long growth season in the UK, from March to November (DEFRA, 2007). *M. giganteus* is an untilled, perennial crop, and after 3-5 years following establishment, shoots are harvested annually, for up to 20 years. Stability of land use and longer growing seasons of *M. giganteus* provides a better habitat, and increased biodiversity, for animals and invertebrates above ground, relative to arable cropping (Haughton, *et al.*, 2009). High growth rates, water efficiency, and carbon inputs, are likely to promote stable microbial communities in the rhizosphere.

1.5 Microbial communities in *Miscanthus giganteus* roots

Bacterial communities in the rhizosphere of *M. giganteus*, may facilitate soil remediation. For example, *Miscanthus* rhizosphere bacterial communities have been shown to remediate soil polluted with polycyclic aromatic hydrocarbons (PAH) (Didier, *et al.*, 2012). Nitrification activity by bacterial and archeal communities has also been detected in the rhizosphere of *M. giganteus* (Mao, *et al.*, 2011). AMF are present in wild *Miscanthus spp.* (An, *et al.*, 2008; Fujiyoshi, *et al.*, 2006; Kojima, *et al.*, 2007; Lee, *et al.*, 2008; Murakoshi, *et al.*, 1998; Saito, *et al.*, 2000). However, the extent to which AMF colonise *M. giganteus*, in UK bioenergy cropping systems, has not been established. Patchy growth and crop

failure, where little or no growth occurs from rhizomes, are major problems for *Miscanthus* growers, and could be due to a number of reasons, particularly poor storage of rhizomes (Pers Com 1. Steve Croxton, Renewable Energy Crops, 2010). However, *M. giganteus* planted in fields previously cultivated with *M. giganteus*, have much higher yields from the first year of growth, and therefore growers suspect something is altered in the soil, such as beneficial microbes (Pers Com 2. Paul Carver, New Energy Farms, 2012). *M. giganteus* bioenergy cropping systems are grown in a monoculture, and receive P and K fertilisers, and therefore AMF colonisation may be discouraged (Lekberg and Koide, 2005). However, *M. giganteus* is a perennial crop, grown in the same field for up to twenty years, with no crop rotations and no tillage. It can shade out competitors, normally does not require N fertiliser in fertile soils, and yield is currently not thought to be majorly affected by pathogens, and therefore there are low inputs of agrochemicals. However, *M. giganteus* is susceptible to a number of cereal pathogens, which means *M. giganteus* may act as a reservoir which could be problematic for nearby cereals or future crop rotations (Glynn, *et al.*, 2014). With no rotations and low chemical inputs, bioenergy cropping systems may encourage a richer diversity of microbes and AMF in the rhizosphere, compared to conventional annual arable cropping systems, as conventional practices have been shown to harbour a lower microbial diversity than organic farms (van der Gast, *et al.*, 2011a). Understanding belowground microbial interactions could help improve *Miscanthus* growth, nutrition, and yields.

1.6 Aims and objectives of the project

The broad aim of this work was to characterise the organization and functional role of arbuscular mycorrhizal fungi, in relation to other rhizosphere fungi, in *M. giganteus* bioenergy cropping systems. The work presented here was split into four parts, to address the following four questions.

- 1) The aim of Chapter 2 was to determine the relative abundance of AMF in *M. giganteus* roots, compared to other rhizosphere fungi, and other plants, as there is currently no information to address the first question:
 - *Are AMF abundant in the roots of M. giganteus, and are they present at similar levels to other herbaceous plant species and land use types?*

2) There is little understanding of what affects the composition of rhizosphere fungi and AMF at small spatial scales. The aim of Chapter 3 was to determine how core and satellite communities of rhizosphere fungi and AMF taxa are organised over space and time, in *M. giganteus* bioenergy cropping systems, to address the second question:

- *Are core and satellite communities of rhizosphere fungi and AMF stable over space and time, and is community composition driven by environmental variables?*

3) It is thought AMF are important for the growth and nutrition of many plants, but there is no understanding of their role in bioenergy cropping systems. Chapter 4 determined the importance of rhizosphere fungi and AMF populations for the growth and nutrition of *M. giganteus*, to address the third question:

- *Are AMF and rhizosphere fungi important for the health and nutrition of *M. giganteus*?*

4) The aim of Chapter 5 was to determine the function of rhizosphere eukaryotes, including fungi and AMF, in *M. giganteus* roots, comparing morning to evening gene expression, to address the fourth question:

- *What is the functional significance of rhizosphere fungi, including AMF, in the fine roots of *M. giganteus*, and are their functions stable diurnally?*

Chapter 2. A meta-analysis of the composition of herbaceous root mycobiomes

2.1 Introduction

2.1.1 Fungal phylogeny

The soil is teeming with microbial life. In one gram of soil there are an estimated 200 m fungal hyphae, which comprises 20 - 30 % of the microbial biomass (Leake, *et al.*, 2004). Soil fungi have many vital ecological roles and interactions with plants and other organisms, as symbionts, commensals, parasites and saprotrophs. Studies have shown there is a selection for microbes in the rhizosphere, as certain communities are more abundant in the rhizosphere than in the bulk soil (Gomes, *et al.*, 2003). Fungi are a kingdom in the Eukarya, and are typically divided into the phyla of the dikarya fungi Ascomycota and Basidiomycota, and the basal phyla Glomeromycota, Zygomycota, Mucoromycotina, Blastocladiomycota, Chytridiomycota, Neocallimastigomycota, Microsporidia and Incertae sedis (which means hard to place) (**Figure 2.1**).

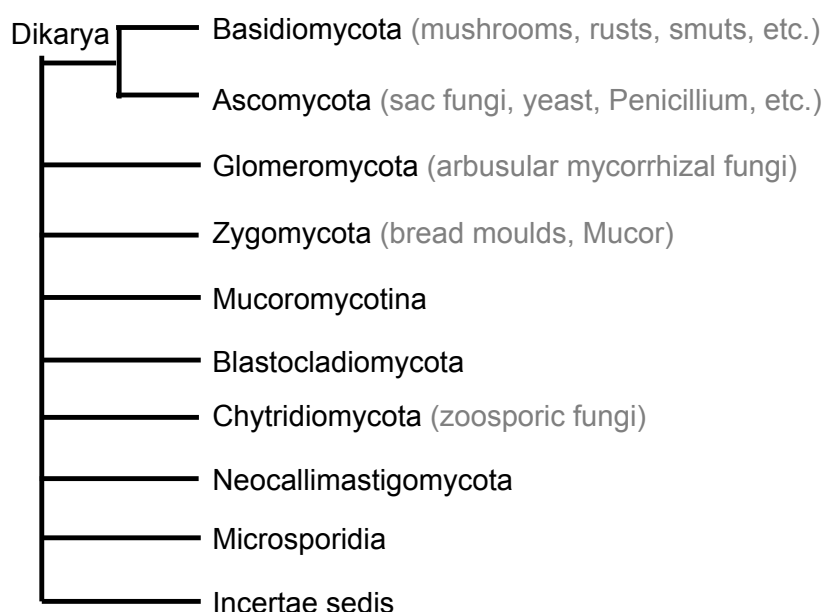


Figure 2.1 Phylogenetic tree of the major fungal phyla, the Dikarya Ascomycota and Basidiomycota, Glomeromycota, and basal fungi Zygomycota, Mucoromycotina, Blastocladiomycota, Chytridiomycota, Neocallimastigomycota, Microsporidia and Incertae sedis. The tree was created using a combination of the UNITE database (Abarenkov, *et al.*, 2010), Tree of Life Web (Blackwell, *et al.*, 2006), and the phylogenetic alignment of 82 fungal genomes (Wang, *et al.*, 2009).

2.1.2 Fungal phyla on roots

Roots interact with many types of fungi, however it is not clear which fungal phyla typically make up the root mycobiome. It is also not clear how this varies between land use types, soil types, and plant families. Historically, to estimate the diversity of fungi in the roots, morphological and isolation techniques have been carried out, including dilution plating and culturing of general soil fungi, and spore counts and root length colonisation of stained roots for arbuscular mycorrhizal fungi (AMF) (Brundrett, *et al.*, 1996; Brundrett and Juniper, 1995). However, many fungi have not been cultured, as there are ~ 70,000 fungal species described and an estimated 1.5 million species total (Hawksworth, 2001), and therefore the use of culture dependent methods alone may under estimate diversity (Liu, *et al.*, 2006). For example, AMF are complicated to study *in vitro* due to their biotrophic and hypogeous lifestyle, and their requirement for a living host plant to grow spore cultures, or more recently *in vitro* root-organ culture (Fortin, *et al.*, 2002), which requires optimization, and AMF are just one group of fungi. Also, the relative abundance of fungi isolated using culture dependent methods may not represent that in the environment.

Fungal abundance and diversity in roots has traditionally been estimated using staining and analysis of hyphae and spore morphology, particularly for analysis of AMF. However, use of hyphae and spore morphology to infer taxonomy requires great expertise, as there are currently 241 Glomeromycota species described (Schüßler and Walker, 2014), and furthermore some AMF, such as *Paraglomus* spp, do not stain and have similar spores to *Glomus* spp., which can render such techniques subjective and problematic (Morton and Redecker, 2001). In addition, the presence of fungal or AMF spores does not necessarily equate to host colonisation. Molecular techniques have been devised to assess fungal and AMF taxonomy and diversity in roots, including methods such as clone libraries, terminal restriction fragment length polymorphisms, and more recently, next generation sequencing, of small and large subunit ribosomal RNA (SSU and LSU rRNA) and the Internal Transcribed Spacer (ITS) region. However, the large majority of molecular research about root fungi has used techniques which assess diversity, such as terminal restriction fragment length polymorphisms (TRFLP) or denaturing gradient gel electrophoresis (DGGE), while very few studies have investigated total rhizosphere fungal taxonomy, using technologies such as next generation sequencing (Allen, *et al.*, 2003;

Lumini, *et al.*, 2010). The aim of this research was to elucidate whether there is a typical root mycobiome, what it is composed of, and how it varies in different plant species and environments.

2.1.3 Literature search and addition of novel mycobiomes

There are a limited number of molecular sequence libraries from roots, which provide taxonomic identity of total fungi (mycobiomes), rather than just community diversity, or specific groups. For example, efforts have been made to collate an online database of described Glomeromycota species <http://www.lrz.de/~schuessler/amphylo/> (Schüßler and Walker, 2014) and there is a database of virtual Glomeromycota libraries, including ecological and environmental categories <http://maariam.botany.ut.ee> (Opik, *et al.*, 2010), but there is no database to represent all fungal phyla within roots. The present study aims to elucidate the fungal phyla that make up the mycobiome of herbaceous plants, typically associated with arbuscular mycorrhizal fungi, rather than orchid, ecto-, or ericales- mycorrhizal hosts. To address this issue, a dual approach was taken, to collate existing knowledge and add more root mycobiomes to the database. First, to collate existing knowledge, a literature search was made for general fungal mycobiomes, from herbaceous hosts mainly associated with AMF. Clone libraries and next generation sequencing libraries were included, as both provide taxonomic and frequency information, and mycobiomes profiled using root DNA, and RNA were distinguished, in order to compare total and active communities respectively. Secondly, eight more mycobiomes were generated, to add to the currently sparse database, to assess mycobiomes in a range of arable and other managed systems.

2.2 Methods

2.2.1 Literature search and database collection

A literature search was carried out using the ISI Web of Science database (1999 - 2014), with search terms root*, fung*, and communit*. A 'mycobiome' was defined as any general fungal sequence library with taxonomic information. Mycobiomes from herbaceous roots were included, from molecular starting materials DNA and RNA. Excluded from analysis were mycobiomes mainly

associated with Orchidaceae (Orchid mycorrhiza) or Ericales (Ericoid mycorrhiza), primers selecting Glomeromycota specifically, and libraries made from cultured isolates. Relevant data was extracted from the collated studies, including molecular starting material (DNA or RNA), the relative abundance of fungal phyla (where necessary relative abundances were calculated from OTU numbers and grouped into phyla Ascomycota, Basidiomycota, Glomeromycota, Chytridiomycota, and other basal groups combined), and potential 'factors'; plant name and family, primers used, photosynthesis type (C3 or C4 in Poaceae, as many Poaceae have evolved C4 compared to other plant families), ecotype (wild, arable, or glasshouse), number of sequences, and number of operational taxonomic units (OTUs).

Altogether there were twenty-eight studies, twenty plant species, and five plant families, including nineteen Poaceae, five Leguminosae, two Asteraceae, one Brassicaceae, and one Solanaceae mycobiome. Poaceae represented by far the largest database, and therefore an additional analysis was carried out on the factors determining fungal community composition within Poaceae samples, to avoid confounding effects of different plant families. All information on the studies used, and potential 'factors', can be found in **Table 2.1** for DNA studies, and **Table 2.2** for RNA studies. Data was analysed using bar charts for comparison of proportions of fungal phyla in each study, and T-tests to test for significance between fungal groups between each factor.

Table 2.1 Information about each study used in the analysis, from the molecular material DNA. Where, photo means photosynthesis type (Poaceae only), and GH stands for glasshouse.

No.	Paper - Author	Plant Name	Plant Family	Photo Ecotype		Primers	No. Seqs	No. OTUs
1	Yuan <i>et al.</i> (2010)	<i>Oryza granulate</i>	Poaceae	C3	Wild	ITS1-F and ITS4	168	31
2	Mouhamadou <i>et al.</i> (2011)	<i>Festuca paniculata</i>	Poaceae	C3	Wild	ITS4 and ITS5	210	73
3	Penton <i>et al.</i> (2013)	<i>Eriophorum vaginatum</i> <i>Vaccinium uliginosum</i>	Poaceae Ericales	C3	Wild	LR3 and LR0R	130,026	743
4	Penton <i>et al.</i> (2013)	<i>Schizachyrium scoparium</i> <i>Sorghastrum nutans</i> <i>Eragrostis curvula</i> <i>Ambrosia psilostachyia</i> <i>Xanthocephalum texanum</i>	Poaceae Poaceae Poaceae Poaceae Poaceae	C4 C4 C4 C3 C3	Wild	LR3 and LR0R	96,669	936
5	Herrera <i>et al.</i> (2013)	<i>Eustachys petraea</i>	Poaceae	C4	Wild	ITS1-F and ITS4	179	10
6	Herrera <i>et al.</i> (2013)	<i>Panicum maximum</i>	Poaceae	C4	Wild	ITS1-F and ITS4	139	12
7	Khidir <i>et al.</i> (2010)	<i>Sporobolus cryptandrus</i>	Poaceae	C3	Wild	ITS1-F and ITS4	158	22
8	Khidir <i>et al.</i> (2010)	<i>Yucca glauca</i>	Poaceae	C3	Wild	ITS1-F and ITS4	116	27
9	Khidir <i>et al.</i> (2010)	<i>Bouteloua gracilis</i>	Poaceae	C4	Wild	ITS1-F and ITS4	173	24
10	Porras-Alfaro <i>et al.</i> (2007)	<i>Bouteloua gracilis</i>	Poaceae	C4	Wild	ITS1-F and ITS4	630	51
11	Neubert <i>et al.</i> (2006)	<i>Phragmites australis</i>	Poaceae	C3	Wild	ITS1-F and ITS4	1,991	105
12	Vandenkoornhuyse <i>et al.</i> (2002)	<i>Arrhenatherum elatius</i>	Poaceae	C3	Wild	AU2 and AU4	49	49
13	Jumpponen and Johnson (2005)	<i>Andropogon gerardii</i> , Panicum virgatum Sorghastum nutans	Poaceae Poaceae Poaceae	C3 C4 C4	Wild	nu-SSU-0817-59 and nu-SSU-1536-39	90	53
14	Smit (1999)	<i>Triticum sp.</i>	Poaceae	C3	Arable	EF4 and EF3	22	17
15	Smit (1999)	<i>Triticum sp.</i>	Poaceae	C3	Arable	EF5 and fungi5	39	16
16	Kwaśna <i>et al.</i> (2010)	<i>Triticum sp.</i>	Poaceae	C3	Arable	ITS1 and M13	87	20
17	Hilton <i>et al</i> (2013)	<i>Triticum sp.</i>	Poaceae	C3	Arable	ITS1-F and ITS4	85	24
18	Gomes <i>et al.</i> (2003)	<i>Zea Mays</i> (Young)	Poaceae	C4	Arable	NS1 and FR1	32	6
19	Gomes <i>et al.</i> (2003)	<i>Zea Mays</i> (Mature)	Poaceae	C4	Arable	NS1 and FR1	55	21
20	Xu <i>et al.</i> (2012)	<i>Pisum sativum</i>	Leguminosae		Arable	ITS1-F and 58A2R	15,540	123
21	Xu <i>et al.</i> (2012)	<i>Pisum sativum</i>	Leguminosae		Arable	ITS1-F and 58A2R		
22	Xu <i>et al.</i> (2012)	<i>Pisum sativum</i>	Leguminosae		Arable	ITS1-F and 58A2R		
23	Yu <i>et al.</i> (2012) a	<i>Pisum sativum</i>	Leguminosae		Arable	ITS1F and ITS2	60,071	142
24	Yu <i>et al.</i> (2012) b	<i>Pisum sativum</i>	Leguminosae		GH	ITS1-F and ITS4	65,067	121
25	Becklin <i>et al</i> (2011)	<i>Taraxacum ceratophorum</i> , <i>Taraxacum officinale</i> , and <i>Polemonium viscosum</i>	Asteraceae Asteraceae Ericales		Wild	nu-SSU-0817-5' and nu-SSU-1536-3'	3,921	230
26	Wehner <i>et al.</i> (2014)	25 Asteraceae sp.	Asteraceae		Wild	ITS1-F and ITS4	156,816	1100
27	Milling <i>et al.</i> (2004)	<i>Solanum tuberosum</i>	Solanaceae		Arable	NS1-GC and NS2	87	5
28	Hilton <i>et al</i> (2013)	<i>Brassica napus</i>	Brassicaceae		Arable	ITS1-F and ITS4	84	25

Table 2.2 Information about each study used in the analysis, from the molecular material RNA (active communities). Where, photo means photosynthesis type.

No.	Author	Plant Name	Common Name	Plant Family	Photo	Ecotype	Primers	Month	No. Seqs	No. OTUs
1	Jumpponen <i>et al.</i> (2011)	<i>Andropogon gerardii</i>	Tallgrass prairie	Poaceae	C3	Wild	nu-SSU-0817-5' and nu-SSU-1536-3'	May	42	30
2	"	"	"	"	"	"	"	July	77	17
3	"	"	"	"	"	"	"	Sep	87	33
4	"	"	"	"	"	"	"	Nov	83	28

2.2.2 Novel fungal microbiomes

To add to the sparse literature, eight more root fungal microbiomes were sequenced (**Table 2.3**). A variety of plant families, from a range of land use types, were represented to expand the range of mycobiomes currently in the database. Mycobiomes from four plant species had not been sequenced previously: *Trifolium repens*, *Glycine max*, *Allium cepa*, and *Miscanthus giganteus*. The two Leguminosae species *G. max* (Gosling, *et al.*, 2013) and *T. repens* (Hazard, *et al.*, 2013), were sequenced to add to the sole Leguminosae species in the database, *Pisum sativum*. *Brassica napus* (Hilton, *et al.*, 2013) and *Zea mays* (Gosling, *et al.*, 2013) were sequenced to compare NGS mycobiomes to clone libraries, of the same plant species from published data. In addition, OSR2 and the *Brassica napus* published clone library (Hilton, *et al.*, 2013), were from the same DNA stock, to directly compare the technical replicates using two technologies. OSR2 was included as another pyrosequenced sample, but with a different crop rotation history, to determine whether crop rotation, or sequencing technology, have more impact on detection of fungal phyla. None of the eight root mycobiomes had been sequenced using next generation sequencing (NGS) previously. NGS was utilised to provide thousands of sequences per sample, compared to traditional clone libraries with Sanger sequencing, which only generate ~80 sequences. The same DNA extraction and sequencing methods were used to enable comparison between these novel mycobiomes. All of the DNA starting material was taken from previous studies briefly described here. For more context for each sample, collection details and environmental variables at study site, see references (Gosling, *et al.*, 2013; Hazard, *et al.*, 2013; Hilton, *et al.*, 2013).

Brassica napus (oil seed rape OSR) was used as a negative control, being a non-mycorrhizal plant, from the Brassicaceae family. OSR1 was taken from a *B. napus* field after three years of winter wheat, and OSR2 was taken from a *B. napus* field after three consecutive years of *B. napus* plantation, to look at the effects of crop rotations on fungal proportions (Hilton, *et al.*, 2013). An AMF pot trial of *Allium cepa* (onion), was used as a positive control, which had been inoculated with a mixture of six Glomeromycota species, and was highly mycorrhizal morphologically (pers. corr. Andrew Taylor, University of Warwick), and expected to have a very high, if not exclusive, AMF microbiome. Field samples of *Trifolium repens* (clover) were used to represent leguminous (N-fixing) unimproved grassland (Hazard, *et al.*, 2013). Field samples of *Zea mays* (maize) were used to represent a C4, annual, arable grass system (Gosling, *et al.*, 2013). Field samples from *Glycine max* (soybean) were used to represent a leguminous (N-fixing) arable system (Gosling, *et al.*, 2013). Finally, field samples from *Miscanthus x giganteus* bioenergy cropping system were used to represent a C4, perennial, arable grass system (Chapter 2). More details on all eight experimental samples are in **Table 2.3**.

Table 2.3 Experimental samples used for characterisation of root fungal microbiomes.

Species	Common name	Plant Family	Location	Date	Ecotype	Reference
<i>Miscanthus x giganteus</i>	Miscanthus	Poaceae	Brattleby, Lincolnshire	Jun-11	Arable	Chapter 2
<i>Miscanthus x giganteus</i>	Miscanthus	Poaceae	Brattleby, Lincolnshire	Oct-11	Arable	Chapter 2
<i>Brassica rapa</i>	OSR	Brassicaceae	East Anglia	Jun-07	Arable	Hilton (2013)
<i>Brassica rapa</i>	OSR	Brassicaceae	East Anglia	Jun-07	Arable	Hilton (2013)
<i>Zea mays</i>	Maize	Poaceae	Wellesbourne, Warwickshire	Jul-07	Arable	Gosling (2013)
<i>Glycine max</i>	Soybean	Leguminosae	Wellesbourne, Warwickshire	Jul-07	Arable	Gosling (2013)
<i>Trifolium repens</i>	Clover	Leguminosae	Republic of Ireland	Aug-06	Unimproved Grassland	Hazard (2013)
<i>Allium cepa</i>	Onion	Amyrillidaceae	Wellesbourne, Warwickshire	Aug-08	Glasshouse	unpublished

2.2.3 DNA extraction and amplicon pyrosequencing

DNA was extracted from 200 mg of washed roots, using a FastDNA SPIN Kit for Soil (Qiagen), according to the manufacturer's instructions, and stored at -20 °C. 18S rDNA fragments were amplified using the fungal primers SSUForward (TGGAGGGCAAGTCTGGTG) and funTitSsuRev (TCGGCATAGTTTATGGTTAAG) (Foster, *et al.*, 2013). 18S rDNA fungal primers were used because the sequence corresponded to clone libraries of Glomeromycota 18S rDNA specific fragments, within the same field using AML1/2 (Lee, *et al.*, 2008), enabling identification of AMF fragments amongst general fungal sequences (Chapter 2). Universal Tails were attached to 5' ends. Universal tails were: forward (GTGTGAAATTGTTACGCT) and reverse (ACTGGCCGTCGTTACAAC), and barcodes, Roche 454 MIDs 1-8. PCR amplification occurred as described in the emPCR Amplification Method Manual (Roche, Branford, USA). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK). Multiplex Identifiers, MID1-8 of the 454Standard MID set, were added to the 5' end during a second round of PCR amplification, to identify samples on the multiplex, and hybridise DNA to the capture beads, respectively, according to the 454 Sequencing Guidelines for Experimental Design 2012 handbook (Roche, 454 Life Sciences Corp., Branford, USA). Unidirectional sequencing, from the forward primer, was carried out on a Roche 454 GS Junior Titanium by a service provider (MicroPathology, Warwick Science Park, UK), following the manufacturer's protocol (Roche, Branford, USA).

2.2.4 Sequence data analysis

SFF files were converted to .txt files, with MOTHUR sffinfo function (Schloss, *et al.*, 2009). All further sequence processing was carried out on Qiime (Caporaso, *et al.*, 2012) default settings, with few exceptions noted. Only sequences between 430 bp and 690 bp were analysed, which represented over 97 % of sequences. Sequences were then denoised using Denoise wrapper 1.4.0 (Reeder and Knight, 2010). Operational Taxonomic Units (OTUs) were picked with 97 % similarity, the default similarity threshold for fungal species, using the uclust picking method (Edgar, 2010). The cluster seed from each OTU was picked for the representative set. Representative OTUs were aligned to the Silva 111 database (Quast, *et al.*, 2013), for eukaryote 18S rDNA, using PyNAST

alignment (Caporaso, *et al.*, 2010). Chimeras were identified from the aligned dataset with ChimeraSlayer (Haas, *et al.*, 2011). Representative OTUs were assigned taxonomy with the RDP classifier (Wang, *et al.*, 2007) to the Silva 111 database, level 5 and level 6. Chimeras were removed and OTU tables were created. All non-fungal species were removed from the OTU table, then relative abundance of each fungal group was calculated, as a percentage of total sequence number.

2.3 Results

2.3.1 Experimental sample sequence numbers

A total of 60,896 raw sequences were obtained from 454-pyrosequencing the eight libraries. There were 1,542 fragments outside bounds of 430-690 bp, 4,321 sequences below the quality score threshold, 1,529 sequences with more than 6 homopolymers, and 37,578 fragments mismatched to more than five primer bases, leaving a total of 15,594 sequences. After denoising, there were 8,698 clusters at 97 % similarity (OTU's), and 166 chimeras were found and removed from analysis. Sequences were aligned to the Silva111 SSU database, however there was a high proportion of contamination. There were 7,935 plant sequences, 277 Holozoa sequences, 86 Rhizaria sequences, 38 Alveolata sequences, 30 Amoebozoa sequences, and 26 other sequences. Only 7,036 sequences were fungal, which was 45.6 % of assigned, and 11.6 % of raw sequences. The number of fungal sequences, per sample, were as follows; 681 in OSR1, 931 in OSR2, 874 in Miscanthus June, 2486 in Miscanthus October, 772 in maize, 253 in soybean, 360 in clover, and 679 in onion. At 97 % similarity level, there were a total of 169 OTUs, with the number of OTUs per sample as follows: 86 in OSR1, 89 in OSR2, 54 in Miscanthus June, 90 in Miscanthus October, 49 in maize, 30 in soybean, 44 in clover, and 10 in onion.

2.3.2 Experimental sample phyla

Ascomycota were found in all samples, with a mean of 68.1 % (**Figure 2.2**). In all sequence libraries, except onion, the Ascomycota were the dominant fungal group, which represented 86.2 % of sequences in soybean, 81.1 % of sequences in OSR1, 75.8 % of Miscanthus October sequences, 74.0 % of

sequences in OSR2, 69.8 % of sequences in Miscanthus June, 61.1 % of sequences in clover, 54.2 % of sequences in maize, and 43.0 % of sequences in onion (**Figure 2.2**). Basal fungi (all fungal phyla except the Dikarya Ascomycota and Basidiomycota, but not including Glomeromycota, which has been analysed separately) were found in all libraries, except Onion, with a mean of 11.2 %. Basal fungi were the second most abundant group in Miscanthus June (25.5%), Miscanthus October (19.3 %), and OSR1 (10.6 %), and present in OSR2 (7.6 %), maize (18.3 %), soybean (2.4 %), clover (6.1 %), but none were found in onion. Glomeromycota were found in all libraries, except OSR2, with a mean of 12.5 %, and were the dominant phyla in onion (57 %). Glomeromycota were the second most abundant group, after Ascomycota, in soybean (6.7 %), and clover (30.0 %), and were found in OSR1 (0.1 %), Miscanthus June (1.83 %), Miscanthus October (2.7 %), maize (1.3 %). Chytridiomycota were found in all libraries, except Onion, were the third most abundant phylum overall, with a mean of 11.2 %, and were the second most abundant group in maize (26.3 %) (**Figure 2.2**). Chytridiomycota were fairly abundant in OSR1 (4.4 %), OSR2 (6.6 %), soybean (4.7 %), and less abundant in clover (1.94 %), Miscanthus June (0.7 %), and Miscanthus October (0.64 %). Basidiomycota were found in five libraries, not including maize, onion, and soybean, with an overall mean of 0.64 %. Basidiomycota were highest in Miscanthus June (2.1 %), and represented 0.9 % in OSR1, 0.3 % in OSR2, 1.0 % in Miscanthus October, and 0.8 % in soybean. Other (unidentified) fungi were found in five libraries, with a mean of 1.9 %, and were highest in OSR2 (11.5 %), followed by OSR1 (2.9 %), Miscanthus October (0.4 %), Miscanthus June (0.1 %), and maize (0.1 %).

2.3.3 Experimental sample class

At the class level four Pezizomycotina groups were abundant across most samples (Sordariomycetes, Leotiomycetes, Dothideomycetes and Eurotiomycetes), and in some samples Glomaceae, Mortierella, *Olpidium brassica*, Zygomycetes, and Chytridiomycetes, were relatively abundant (**Figure 2.2**). Sordariomycetes were the most abundant class overall, with a mean of 33.8 %, and were the dominant class in OSR1 (42.2 %), OSR2 (41.6 %), Miscanthus October (27.8 %), and soybean (48.6 %), but still relatively high in Miscanthus June (21.1 %), maize (22.6 %), clover (25.6 %), and onion (40.9 %). Leotiomycetes were the second most dominant class after Sordariomycetes,

had an overall mean of 14.5 %, and were present in all samples. The highest abundance of Leotiomyces was in soybean (26.7 %), followed by clover (21.2 %), Miscanthus October (17.1 %), OSR1 (15.3 %), Miscanthus October (14.6 %), maize (14.5 %), OSR2 (5.9 %), and onion (0.3 %). Glomaceae were the third most abundant taxon overall (11.3 %), were the dominant group in onion (53.8 %), and clover (27.0 %), were fairly abundant in soybean (6.9 %), and had the third largest mean of 11.3 % across all samples. However, the remaining five samples had relatively low proportion of Glomaceae, 0.0 % in OSR1, 0.1 % in OSR2, 1.3 % in Miscanthus June, 1.0 % in Miscanthus October, and 0.9 % in maize. Dothideomycetes were the fourth overall dominant class, with a mean of 11.3 %, and were present in all samples. The abundance of Dothideomycetes was highest in Miscanthus June (21.2 %), followed by OSR2 (15.7 %), OSR1 (12.6 %), maize (12.1 %), Miscanthus October (11.5 %), soybean (8.8 %), clover (7.5 %), and onion (0.9 %). *Mortierella* were the fifth most abundant taxon overall (6.9 %), and was the dominant taxon in Miscanthus June (23.2 %), fairly abundant in Miscanthus October (17.1%), maize (5.6 %), OSR1 (3.9 %), and OSR2 (3.9 %), but were lowly represented in soybean (0.4 %), clover (1.4 %), and onion (0.0 %). Eurotiomycetes was the sixth most abundant class, present in all samples, with an overall mean of 6.9 %. The abundance of Eurotiomycetes was highest in Miscanthus October (17.8 %), followed by Miscanthus June (10.5 %), OSR1 (6.7 %), OSR2 (5.8 %), maize (4.2 %), clover (1.9 %), soybean (1.2 %), and onion (0.4 %). An uncultured Chytridiomycete was the seventh most abundant taxon, with a mean of 4.2 %, and was the dominant taxon in maize (25.3 %). The uncultured Chytridiomycete was present in OSR1 (2.2 %), OSR2 (1.3 %), soybean (3.2 %), and clover (1.7 %), but was absent in Miscanthus June, Miscanthus October, and onion (**Figure 2.3**). *Olpidium brassica* was only present in OSR1 (3.0 %), and OSR2 (11.5 %). *Zygomycete sp .AM2008a* was fairly abundant in maize (8.7%), and present in soybean (0.8 %), and Miscanthus June (0.1%). Pezizomycetes were present in clover (3.6 %), OSR2 (1.7 %), OSR1 (1.5 %), soybean (0.8 %), Miscanthus June (0.2 %), Miscanthus October (0.1 %). Diversispora were present in onion (3.2 %), and maize (0.1 %). The remaining taxa were only present rarely and occasionally (**Figure 2.3**).

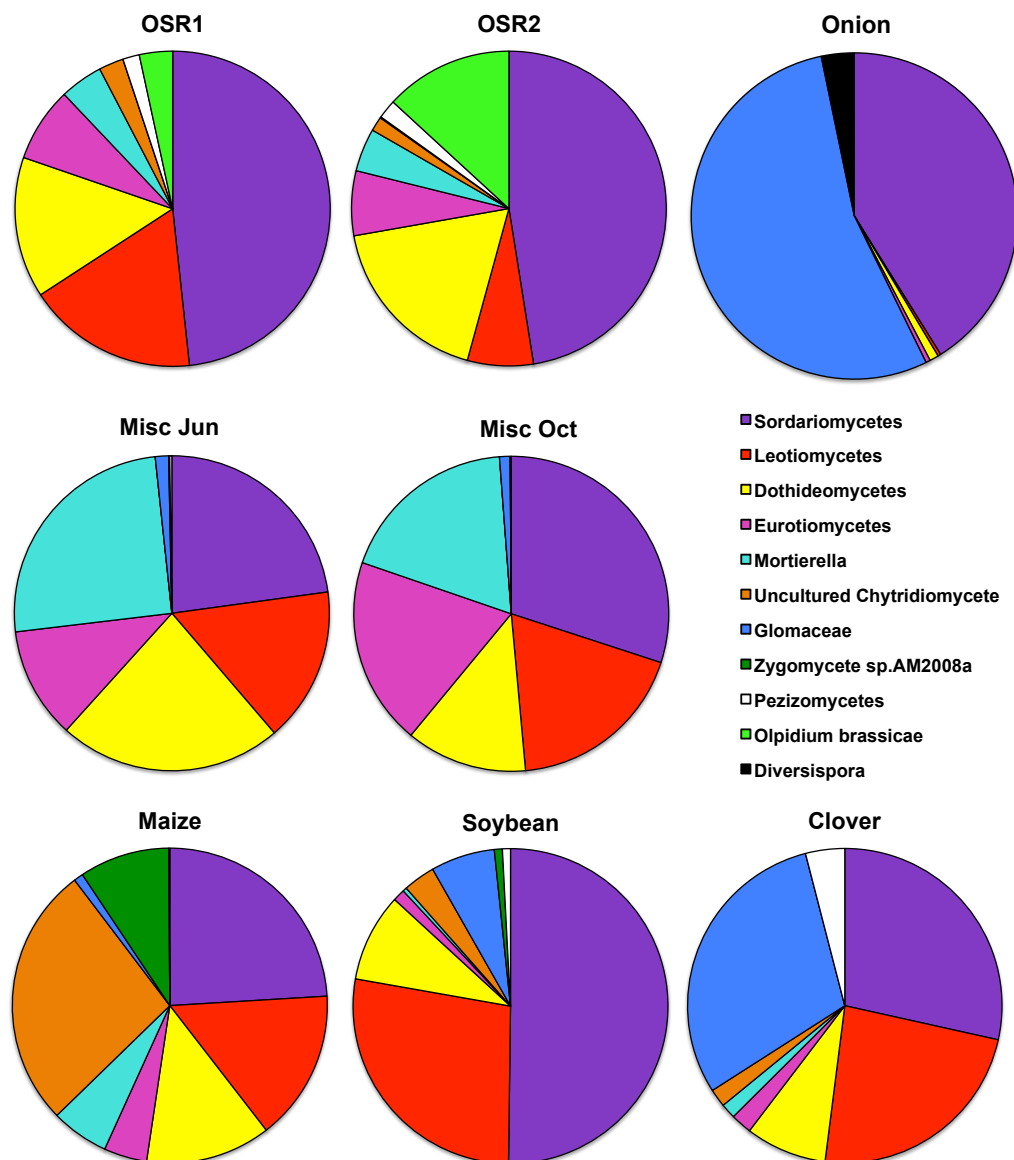


Figure 2.2 Relative abundance of the top eleven fungal classes overall, from the eight experimental root samples. The total percentage of all sequences covered in the pie charts are OSR1 87.4 %, OSR2 87.5 %, Miscanthus June 92.2 %, Miscanthus October 92.5 %, Maize 93.9 %, Soybean 96.8 %, Clover 90.0 %, Onion 99.6 %. Note, *Olpidium brassicae* was only found in oil seed rape.

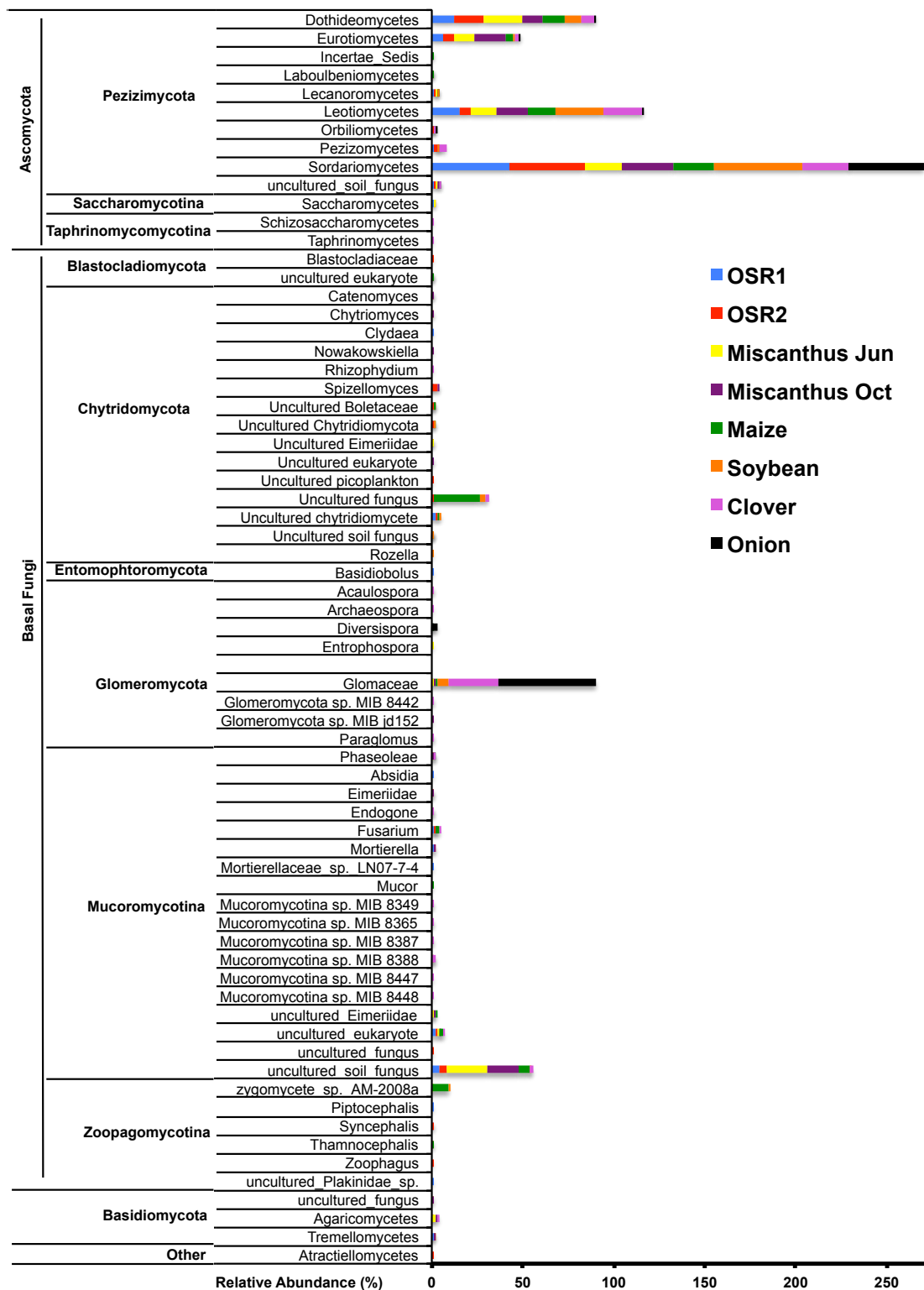


Figure 2.3 Relative abundance of all fungal taxa in the eight experimental root microbiomes.

2.3.4 Literature review root mycobiomes from DNA

A literature review was performed, which identified twenty eight herbaceous root mycobiomes for the analysis. The majority of publications found were libraries from the Poaceae plant family (nineteen), but also represented were five Leguminosae, two Asteraceae, one Brassicaceae, and one Solanaceae mycobiomes (**Table 2.1**). The Poaceae mycobiomes were from a range of semi natural systems; wild rice (Yuan, *et al.*, 2010), permafrost (Penton, *et al.*, 2013), sub alpine grassland (Mouhamadou, *et al.*, 2011), tall grass prairie (Jumpponen and Johnson, 2005; Penton, *et al.*, 2013), semi-arid grassland (Khidir, *et al.*, 2010), common reeds (Neubert, *et al.*, 2006), tall oat grass (Vandenkoornhuyse, *et al.*, 2002), desert grass (Porrás-Alfaro, *et al.*, 2007), and arable systems; wheat (Kwasna, *et al.*, 2010; Smit, 1999) and maize (Gomes, *et al.*, 2003). The remaining hosts represented were from arable systems: pea (Leguminosae) (Xu, *et al.*, 2012; Yu, *et al.*, 2012a; Yu, *et al.*, 2012b), oilseed rape (Brassicaceae) (Hilton, *et al.*, 2013), and potato (Solanaceae) (Milling, *et al.*, 2004), and two semi natural Asteraceae systems, dandelions (Becklin, *et al.*, 2012), and an Asteraceae mix (Wehner, *et al.*, 2014). From these papers, OTU numbers were turned into relative abundance and grouped into phyla, based on taxonomy (**Table 2.1, Figure 2.4**).

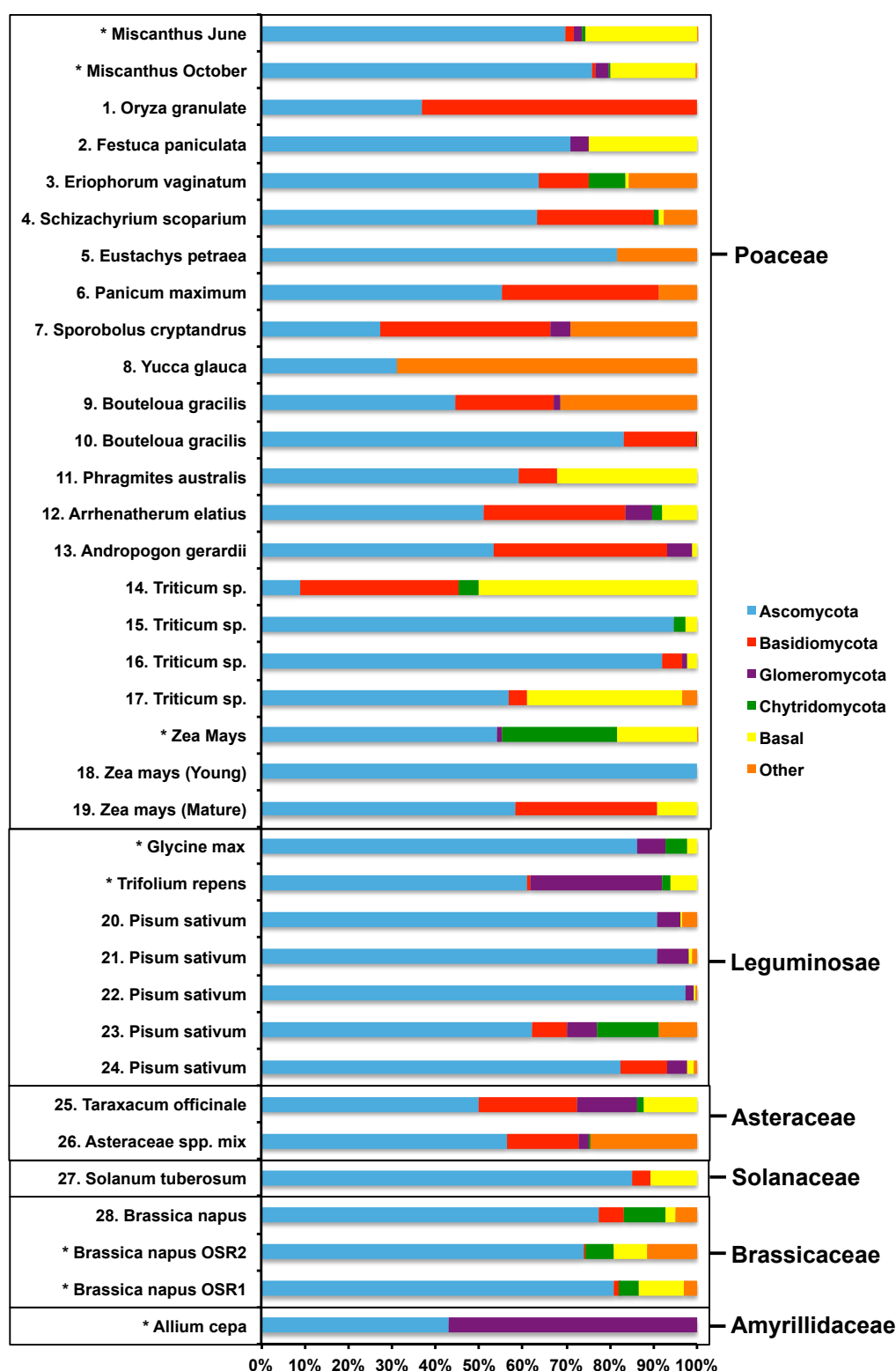


Figure 2.4 Relative proportions of fungal phyla sequences, where ‘Other’ represent sequences with no database match, from the eight new mycobiomes (marked with *), and the twenty-eight past studies from the literature search, numbered 1-28. See **Table 2.1** for more information on the published studies, and see **Table 2.3** for information on the eight new libraries from this study. Plant hosts are separated by plant family. Basal fungi include all basal fungi except Glomeromycota and Chytridiomycota which were analysed separately.

It is unfortunate that no studies had biological or technical replicates, and there were few hosts for which more than one sequence library was available, so no understanding of variability within plant species. This could be due to the expense and time required to produce replicates, and the staggering number of plant species left to sequence. However, the data does give us an idea of the overall proportions of fungal phyla on herbaceous plant roots (**Figure 2.4**).

2.3.5 Experimental samples and literature review combined

Across the thirty six mycobiomes, combining the published studies with the eight new mycobiomes presented here, Ascomycota were the dominant phyla, followed by Basidiomycota, basal groups, other (unidentified) fungi, Glomeromycota and Chytridiomycota (**Figure 2.4**). Ascomycota had a mean of 65.8 % (\pm 3.6 SEM) relative sequence abundance, and ranged from 9.1 % to 100 %, and were dominant in thirty-one studies out of thirty-six. Basidiomycota had a mean relative abundance of 12.5 % (\pm 2.7 SEM), ranged from 0.0 % to 63.0 %, and were the dominant phyla in two studies, of *Sporobolus cryptandrus* (Khidir, *et al.*, 2010) and *Oryza granulate* (Yuan, *et al.*, 2010). Basal fungi had a mean relative abundance of 7.9 % (\pm 2.0 SEM), ranged from 0.0 % to 50.0 %, and were dominant in one study, of *Yucca glauca* (Khidir, *et al.*, 2010). Other (unidentified) fungi had a mean relative abundance of 6.7 % (\pm 2.3 SEM), ranged from 0.0 % to 69.0 %, and were dominant in one study, of wheat (Smit, 1999). Glomeromycota had a mean relative abundance of 4.6 % (\pm 1.8 SEM), and ranged from 0.0 % to 57.0 %, and was dominant in one library, of the onion pot experiment with Glomeromycota inoculation. Chytridiomycota had a mean relative abundance of 2.5 % (\pm 0.9 SEM), and ranged from 0.0 % to 26.3 %.

There was a high degree of variability between the samples, even within the same plant species and study site. For example, *Zea mays* at different life stages had very different profiles, young maize being entirely Ascomycota (**Figure 2.4** 18), whereas the mature maize was composed of Ascomycota, Basidiomycota (**Figure 2.4** 19), and basal fungi. In addition the *Zea mays* sample from the present study, had a large proportion of Chytridiomycota (26.3 %), and Glomeromycota (1.29 %), which were not present in previous maize studies, and no Basidiomycota, unlike the other mature maize sample (**Figure 2.4** No 19 (Gomes, *et al.*, 2003)).

The primer set may have an impact on the mycobiome detected, for example, in wheat the proportion of Ascomycota ranged from 9.1 % to 94.9 %. While three wheat samples, amplified with EF5 – fungi5, ITS1 and M13, and ITS1-F and ITS4, were dominated by Ascomycota, with relative proportions of 94.9 %, 92.0 %, and 56.7 % respectively (**Figure 2.4** 15, 16, 17), a wheat sample amplified with EF4 - EF3 had a very low proportion of Ascomycota, 9.1 %, higher proportions of Basidiomycota (36.4 %) and Chytridiomycota (4.5 %), but was dominated by Basal fungi (50.0 %) (**Figure 2.4** 14) (Smit, *et al.*, 2009).

Another example of variation within plant species, was the five pea mycobiomes (**Figure 2.4** 20-24), which showed large variation in the proportions of Ascomycota, Basidiomycota, Chytridiomycota, and other basal fungi. One reason may be the primers used, but they are also likely to differ due to differences in sampling time and location. In **Figure 2.4**, *Pisum sativum* studies No. 20, 21, and 22 were from different fields, but used the same primers, ITS1F and 58A2R, and had very similar profiles, (mean relative abundance \pm SEM) Ascomycota (93.1 % \pm 2.1), Basidiomycota (0.0 % \pm 0.0), Glomeromycota (4.8 % \pm 1.5), Chytridiomycota (0.0 % \pm 0.0), Basal groups (0.5 % \pm 0.2), and other (1.6 % \pm 0.9) (Xu, *et al.*, 2012). Whereas study No. 23 *Pisum sativum* used primers ITS1F and ITS2 (Yu, *et al.*, 2012a), and compared to ITS1F and 58A2R (Xu, *et al.*, 2012), amplified 30 % less Ascomycota (relative abundance 62.2 %), Basidiomycota were detected (8.2 %) and Chytridiomycota were detected (14.2 %). Finally, study No. 24 (from **Figure 2.4**) *Pisum sativum* used ITS1F and ITS4 (Yu, *et al.*, 2012b), and were similar to previous studies, composed of Ascomycota (82.6 %), Basidiomycota (10.7 %), Glomeromycota (4.3 %), Chytridiomycota (0.0 %), Basal groups (1.6 %) and other (0.8 %). However there were similarities within pea libraries, even though they were all sampled from different locations; all five pea libraries were dominated by Ascomycota, which ranged from 62.6 % - 97.3 % relative abundance, and all contained Glomeromycota (2.2 – 7.21 %), therefore there was some consistency across primer sets in pea (**Figure 2.4**).

Primer set may not have been the only factor affecting community proportions, as the three *Brassica napus* samples used ITS1-F and ITS4, and the clone library (Hilton, *et al.*, 2013) and OSR2 were from the same DNA stock, and still exhibited variation. The two pyrosequenced samples, OSR1 and OSR2,

detected only a small proportion of Basidiomycota, 0.9 % and 0.3 % respectively, whereas the clone library was composed of 6.0 % Basidiomycota (Hilton, *et al.*, 2013). There was a higher proportion of Basal fungi in OSR1 and OSR2, 10.6 % and 7.6 % respectively, compared to the clone library, with 2.4 % Basal fungi. There was also a higher proportion of Chytridiomycota in the clone library, 9.5 %, compared to OSR1 and OSR2, which had 4.4 % and 6.6 % respectively. There were similar proportions of Ascomycota, Glomeromycota, and other fungi, however OSR1 and OSR2 communities were more similar than OSR2 and *Brassica napus*, the technical replicates.

The most important, and surprising, finding from the literature review was the low relative abundance of Glomeromycota sequences. In fact, Glomeromycota were not the dominant fungi in any of the DNA libraries (**Table 2.1**), and were consistently lower than Ascomycota, and often Basidiomycota and basal fungi.

2.3.6 Root mycobiomes from RNA (active communities)

There was only one study that used RNA as a starting molecular material, to analyse active mycobiomes (**Figure 2.5**), a study of tall grass prairie at four time points in the year (Jumpponen, 2011). Ascomycota were most dominant phyla, which was the same as DNA studies described previously. However, the Ascomycota dominance was much lower in RNA samples, representing 47.1 % mean relative abundance, compared to 65 % using DNA. There was a higher proportion of Basidiomycota (23.5 % mean relative abundance), and a similar proportion of basal fungi (6.9 % mean relative abundance) in RNA compared to DNA libraries. Most strikingly, there were nine fold more Glomeromycota (mean relative abundance %) in RNA (22.6 %), compared to overall DNA (2.5 %) (TTEST $P < 0.001$ *). There were also four times more Glomeromycota in the tall grass RNA library (22.6 %) compared with a similar tall grass prairie library, produced in an earlier study with the same primers, but amplifying DNA (5.6 %) (Jumpponen and Johnson, 2005). No other fungal phyla were significantly different between RNA and DNA.

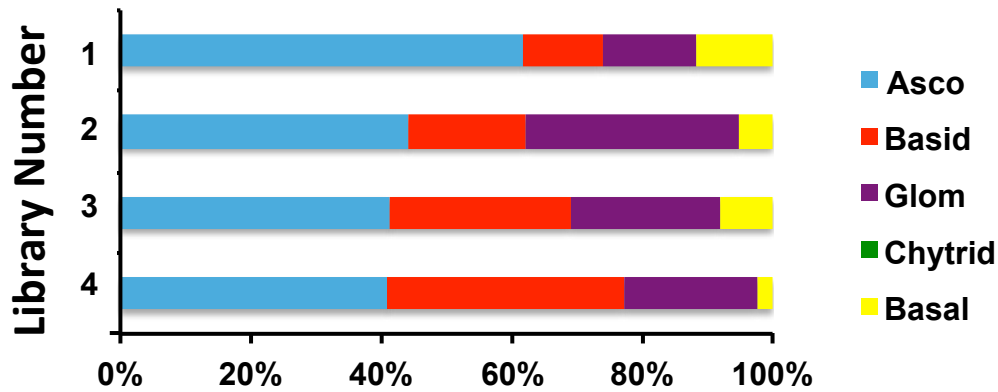


Figure 2.5 Relative proportions of fungal phyla sequences, from RNA studies (**Table 2.2**), Asco (Ascomycota), Basid (Basidiomycota), Glom (Glomeromycota), Chytrid (Chytridiomycota), and Basal (other basal lineages, not including Glomeromycota or Chytridiomycota), and Other (unclassified or no database match).

2.3.7 Mycobiomes of plant families

All fungal phyla were present in each plant family, except Solanaceae and Brassicaceae, but this varied greatly (**Figure 2.2** and **2.4**). The four plant families analysed appear to have different proportions of fungal groups (**Figure 2.4**). The proportion of Ascomycota was highest in Solanaceae (85.0 %), followed by Leguminosae (81.6 %), Brassicaceae (77.5 %), Poaceae (60.7 %), Asteraceae (53.1 %), and Amyrillidaceae (43.0 %). The proportion of Basidiomycota was highest in Asteraceae (19.4 %), followed by Poaceae (17.8 %), Solanaceae (4.5 %), Leguminosae (2.8 %), Brassicaceae (2.4 %), and none in Amyrillidaceae. The proportion of Glomeromycota sequences was highest in Amyrillidaceae (57.0 %), followed by Asteraceae (8.1 %), Leguminosae (8.9 %), Poaceae (1.4 %) and none in Solanaceae or Brassicaceae. The proportion of Chytridiomycota was highest in Brassicaceae (6.8 %), followed by Leguminosae (3.0 %), Asteraceae (1.1 %), Poaceae (2.2 %), and none in Solanaceae or Amyrillidaceae. The proportion of basal fungi was highest in Solanaceae (10.5 %), followed by Poaceae (9.3 %), Brassicaceae (6.9 %), Asteraceae (6.0 %), Leguminosae (0.8 %), and none in Amyrillidaceae. The proportion of other (unidentified) fungi was highest in Asteraceae (12.3 %), followed by Poaceae (8.6 %), Brassicaceae (6.4 %), Leguminosae (2.0 %), and none in Solanaceae or Amyrillidaceae.

It was intriguing that land use type, DNA region, and photosynthesis type, had no statistical impact on differences between any fungal group, only plant family had any significance. Leguminosae had a higher Glomeromycota (8.9 %) and Ascomycota (81.6 %) proportion than Poaceae (1.4 % and 60.7 %, respectively), and lower Basidiomycota (2.8 % compared to 17.8 % in Poaceae), in the literature and experimental samples combined (TTESTs $P = 0.0015$ *, $P = 0.035$ *, and $P = 0.005$ % *, respectively). The proportion of Glomeromycota in Poaceae was also significantly lower than in Asteraceae (TTEST $P = 0.002$ *). There was also significantly higher Basidiomycota (TTEST $P = 0.003$ *) and lower Ascomycota (TTEST $P < 0.033$ *), in Asteraceae, compared to Leguminosae (**Figure 2.4**). However, none of the other differences in fungal phyla between plant families were significant, excluding Solanaceae, Amyrillidaceae, and Brassicaceae as the sample sizes were too small ($n = 1$).

2.3.8 Poaceae mycobiomes

Within the Poaceae plant family, there was large variability between mycobiomes (**Figure 2.6**). However, there were no significant differences in Poaceae samples, between 18S rDNA and ITS DNA, arable and wild, or C3 and C4.

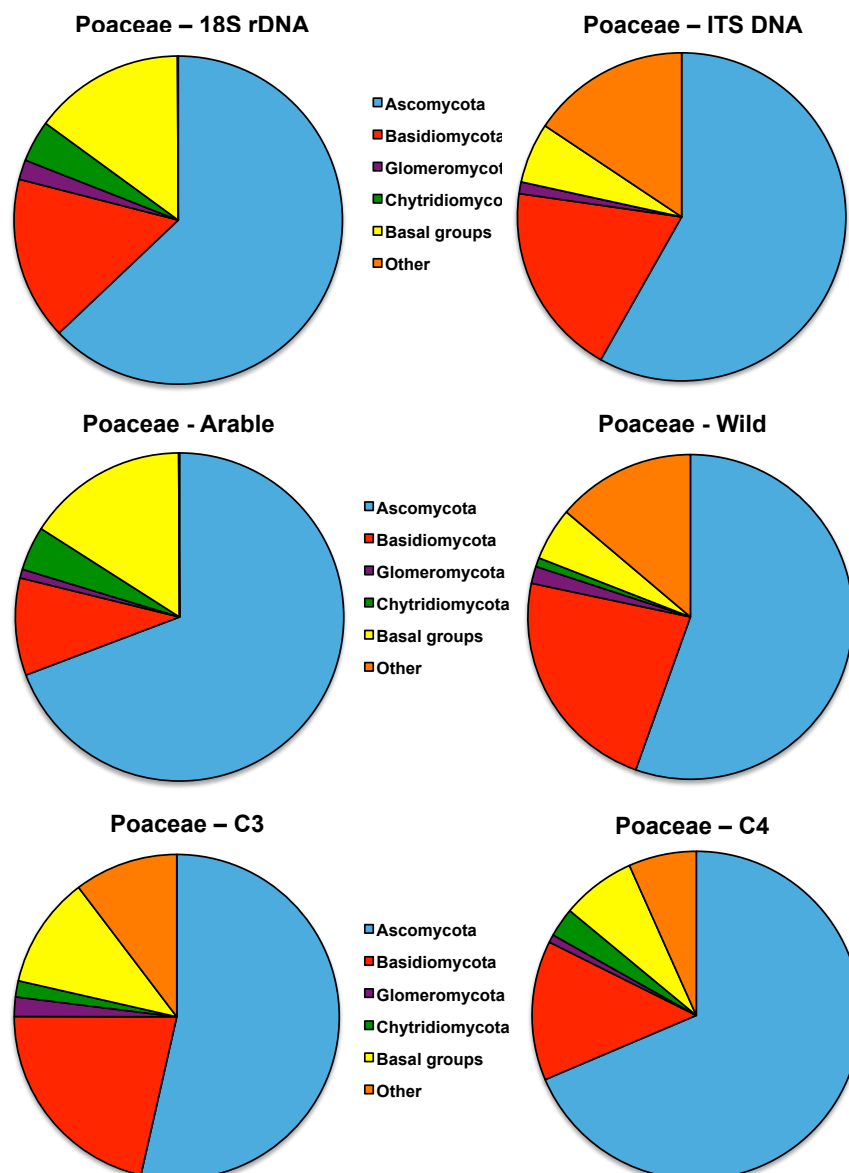


Figure 2.6 Root mycobiomes, mean relative abundance, taken from Poaceae plant family 18S rDNA ($n = 9$), ITS rDNA ($n = 10$), Arable ($n = 8$), Wild ($n = 13$), C3 ($n = 11$), C4 ($n = 10$).

2.4 Discussion

Root mycobiomes were widely variable, but some fungal groups were common in roots, whereas other groups were occasionally very frequent but mostly rare across samples. Ascomycota were the dominant phyla in the vast majority of mycobiomes, and Basidiomycota were also very common, overall. In particular four classes of Ascomycota were common in all experimental samples, Sordariomycetes, Letiomycetes, Dothiomycetes, and Eurotiomycetes, which suggests these could be generalist root inhabitants. Chytridiomycota and other

basal groups including Zygomycota and Mucorales were unexpectedly abundant in some samples, which indicates specialist association with certain hosts, for example as plant parasites (Benny, *et al.*, 2001). However, the Glomeromycota had very low abundance and frequency across studies, which was a surprise, as the vast majority of root fungal research is about Glomeromycota, and they are believed to be important for plant nutrition and growth (Smith and Read, 2008).

2.4.1 Ascomycota

The Ascomycota, or sac fungi, globally represent approximately 75 % of fungi, and are a very general group, united by formation of ascus, which hold ascospores (Hibbett, *et al.*, 2007). They can be necrotrophic or biotrophic, and play many roles which include as saprophytes degrading organic matter, commensals, mutualists with plants, for example *Tuber melanosporum* (truffles), mutualists with algae, making up most fungal partners of lichen, and mutualists with arthropods, and parasites, including *Aspergillus flavus* (aflatoxin) which is carcinogenic to animals and *Chryphonectria parasitica* which cause disease in chestnut trees (Alexopoulos, *et al.*, 1996). Some well known examples of the Ascomycota, include *Saccharomyces cerevisiae* (bakers yeast), *Morchella esculentum* (morels), *Penicillium chrysogenum* (penicillin).

The Ascomycota classes, Sordariomycetes, Leotiomycetes, Dothideomycetes, and Eurotiomycetes (**Figure 2.7**), were found in all samples. The four classes cover an extensive range of ecological functions and genera. The Sordariomycetes contain more than 600 genera, and 3000 species, and are one of the largest Ascomycota classes (Kirk, *et al.*, 2001). The Sordariomycetes function in most ecosystems, as pathogens of plants, mammals, arthropods and fungi, saprotrophs, and endophytes in plants. Some notable species in the Sordariomycetes are the model organism *Neurospora crassa*, *Cryphonectria parasitica* (chestnut blight), and *Magnaporthe grisea* (rice blast). The Letiomycetes consist of 21 families, 510 genera (Hibbett, *et al.*, 2007), and also cover a broad range of ecological roles. The Eurotiomycetes are composed of three subclasses, which include species which produce useful secondary metabolites, toxins, food products, enzymes, and the group contains the model organism *Aspergillus nidulans* (Geiser, *et al.*, 2006). Ecological functions within the Eurotiomycetes, include lichenised groups, and pathogens of humans and

animals (Geiser, *et al.*, 2006). Dothideomycetes are one of the largest and most diverse fungal groups, and their main ecological roles are as pathogens, epiphytes, and endophytes, of living and dead plants (Kirk, *et al.*, 2001). Their pathogenicity in agricultural plants has been the main focus of research (Ohm, *et al.*, 2013), such as the corn pathogen *Cochliobolus heterostrophus*. Dothideomycetes are also partners in lichen associations, and can parasitise fungi and other organisms (Kirk, *et al.*, 2001).

The four ubiquitous Pezizomycotina classes represent a relatively small component of total Ascomycota diversity (**Figure 2.7**), which highlights the importance of these groups in the rhizosphere. However, the extensive ecology and taxonomy of these classes makes it difficult to comment on their likely roles in these systems. The identification of many sequenced fragments did not reach past class level, particularly for Ascomycota, which is likely due to the limited hypervariation within the 18S rDNA region, the short fragments required for 454 pyrosequencing, and the limited number of fungal sequences in the 18S Silva111 database. However, it does give us a useful insight into the commonly found groups within roots.

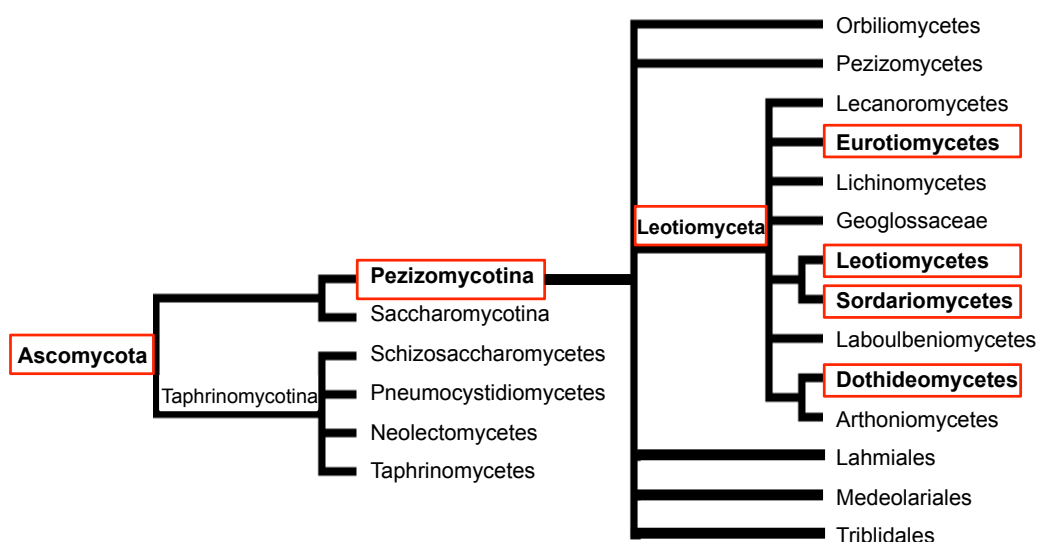


Figure 2.7 Phylogenetic tree of the fungal phylum Ascomycota, divided into sub-phyla, and further divided into the Pezizomycotina classes. Highlighted are the four classes of fungi found in all experimental samples (see **Figure 2.4** for relative abundances). The tree was adapted from Blackwell, *et al.* (2006).

2.4.2 Basal fungi

Basal fungi also contributed consistently to rhizosphere mycobiomes, and occasionally abundantly so. For example Chytridiomycota and Zygomycota species made up a third of the mycobiome in maize, and a *Mortierella* species made up a quarter of the *Miscanthus* mycobiome (**Figure 2.4**). The Chytridiomycota phyla are zoospore forming and flagellated fungi, and consist of around 1000 species (James, *et al.*, 2006). The Chytridiomycota are diverse and ubiquitous, in aquatic and terrestrial habitats. Their main function in soils is thought to be as saprotrophs, degrading chitin, keratin, and cellulose, and as obligate plant pathogens, for example *Synchytrium* is a large genus of pathogens of moss, ferns, and angiosperms, such as potato (James, *et al.*, 2006). The Zygomycota are one of the most understudied but diverse groups, which represent around 1 % (~ 900 species) of the described fungal species, and distinctively reproduce with Zygospores (Benny, *et al.*, 2001; Kirk, *et al.*, 2001). They are common in aquatic and terrestrial habitats, and act as saprobes (including as economically important fruit rots), mutualists, commensals of arthropod stomachs (Harpellales), and parasites of animals, plants, and fungi (Benny, *et al.*, 2001). *Mortierella spp.* are not known to cause disease in animals, plants, or humans, and are useful for a number of industrial and agricultural applications, including as biocontrol agents against nematodes (Al-Shammari, *et al.*, 2013), and in producing lipid based products including biodiesel (Wang, *et al.*, 2011a). Again, it is difficult to assign ecological roles to the basal fungi, which made up large proportions of some roots, as their roles are so diverse in nature. However, their importance may be underestimated in plant roots, as there is limited information on these groups in this habitat.

2.4.3 Glomeromycota

Glomeromycota were very low across different hosts, primer sets, and land use types. The low abundance of Glomeromycota may not be due to primer bias, as twelve different primer sets were used across studies, and Glomeromycota were picked up but at consistently low relative abundance. One explanation may be the intraspecific variation of Glomeromycota, which exhibits up to 20 % divergence in a single multinucleate spore, and is thought to be the cause of low copy numbers of AMF SSU (Hijri and Sanders, 2005). For example, some SSU genotypes clustering at 97 % similarity represent many species, and sometimes

many genotypes are present in a single strain, which makes it difficult to assign taxonomy and estimate abundance of AMF species (Kruger, *et al.*, 2012). Even the model organism *Rhizophagus intraradices* was misclassified due to the heterokaryotic nature of AMF spores and cells (Stockinger, *et al.*, 2009). While searching for the best universal fungal primer set, Schoch, *et al.* (2012) decided to leave out the Glomeromycota from analysis, as the ITS region was too highly variable, meaning that AMF phylotypes could not be accurately affiliated to species. Even though species level taxonomy may not be possible with ITS, assigning to family level should be more accurate, and would be expected to show up in these libraries. In addition, the samples sequenced in the present study were of the SSU, the region recommended for AMF research, and similar proportions of AMF were found in SSU, compared to ITS literature (i.e. no statistical differences).

The lifestyle of AMF may partly explain their low abundance in mycobiomes, since they mainly grow within the root cortex. Therefore, Glomeromycota DNA may not be as easily accessible for amplification as fungal DNA in the rhizoplane, as external AMF hyphae are snapped and washed away before extraction. Therefore the extraction method could lead to variability in endophyte abundance. The molecular starting material had a huge impact, where RNA samples had nine fold higher proportion of Glomeromycota than DNA. This means there could be more Glomeromycota activity in the roots than the DNA suggests, as there may be a lot of inactive fungi, in or on the roots, which are amplified more readily in the PCR, or spores which contain high genome copy numbers. The lack of RNA samples in the meta-analysis highlights the gap in knowledge of AMF activity in the roots, since only one paper was found (Jumpponen, 2011). Alternatively, the extraction method again could have been a factor, as to extract RNA required freezing and complete pulverization of the roots (Jumpponen, 2011), whereas to extract DNA from roots normally involves using a bead beater method which only loosens roots (Gosling, *et al.*, 2013). The only DNA sample with similarly large quantities of Glomeromycota as RNA from tall grass, clover, also used pulverization of roots to extract DNA (Hazard, *et al.*, 2013), similar to the RNA extraction method. However, Glomeromycota still only made up ~ 20 % of grass and clover root activity, with full pulverization.

The importance of Glomeromycota may have been overestimated by experimentally inoculating hosts in the past. Mycobiomes from the AMF

inoculation of onion, were shown here to be comprised of 57 % Glomeromycota, and only 43 % Ascomycota, mostly Sordariomycetes, and no other fungal phyla were present. This proportion of AMF was twenty times higher than mycobiomes in natural and arable systems, since the mean Glomeromycota in literature studies and the novel sequence libraries presented here was 3.1 %, and for Ascomycota 66.4 %. Therefore, this extremely high proportion of Glomeromycota cannot be a realistic assessment of the role of Glomeromycota in nature, as it faces no competition or interaction with the common inhabitants, such as Leotiomycetes, Dothideomycetes, Eurotiomycetes, Basidiomycota, Chytridiomycota and Zygomycota. Representing the true fungal diversity of roots should be prioritised when assessing the ecological role of Glomeromycota.

The present review highlights the need for more taxonomic and functional sequencing of root mycobiomes, particularly meta-transcriptomics, to understand the structure and function of root inhabitants, and to put the Glomeromycota in context of whole rhizosphere functioning.

2.4.4 Leguminaceae and Poaceae mycobiomes

The Poaceae and Leguminosae species had very different mycobiomes, which is interesting as legumes have nitrogen-fixing bacteria associated with their roots, which may alter microbial interactions in their rhizosphere. Also, it was surprising that the relative abundance of AMF in legumes was higher than in grasses. However, it has been suggested that N-fixing bacteria in legumes may encourage other symbionts, which is driven by a byproduct of N₂ fixation, H₂ (Dong, *et al.*, 2003), and this work supports that theory. It would be more robust to have more legume studies to compare with Poaceae. This research highlights the gap in knowledge of whole fungal mycobiomes, in the Leguminosae, and other plant families. It also highlights the importance of the eight additional mycobiomes produced in this study, to add to our currently limited database.

Chapter 3. Spatial and temporal scaling of root fungal and Glomeromycota, core and satellite communities, in a bioenergy cropping system

3.1 Introduction

3.1.1 Biogeography of Microbes

One of the fundamental objectives of ecology is to understand how biodiversity is distributed and maintained across space and time (Oliver, *et al.*, 2012). Understanding the spatial and temporal structure of communities is an essential aim in ecology, as these patterns affect ecosystem functioning. Spatial and temporal variation of organisms is thought to be very common, as environmental and evolutionary factors change through time and space. However, the general principles of microbial biogeography are poorly developed, and there is a growing body of research aimed to understand whether microbial distributions are under the same environmental influences as macro-organisms (Martiny, *et al.*, 2006). There is a theory that “everything is everywhere, but, the environment selects” (Baas Becking, 1934). Testing this principle, ecologists have aimed to discover whether microbial structure and dispersal is random, ubiquitous, or under the influence of biotic and abiotic factors. Studies have been conducted at various spatial scales, including across continents (Lauber, *et al.*, 2009) and landscapes (Griffiths, *et al.*, 2011), down to soil pore level (Ruamps, *et al.*, 2011).

Microbes undergo processes including colonisation, succession, immigration, and extinction, due to factors including selective pressures, competition, and stochastic events (Magurran, 2007; Preston, 1960; van der Gast, *et al.*, 2008). Hanson, *et al.* (2012) hypothesised that the underlying processes which interplay to cause microbial biogeographical patterns are selection, dispersal, drift, and mutation.

Most biogeographical studies analyse spatial or temporal scaling alone, with few considering both together (Oliver, *et al.*, 2012). However, if life displays such dynamic temporal and spatial variation, these variables should be considered

together, and their interactions elucidated. For example, Bell (2010) investigated temporal and spatial patterns of re-colonising bacterial communities in sterile tree holes. Communities grew increasingly different with space over 150 m apart, and time (Bell, 2010). If all communities are constantly changing, a single sample of life is effectively only a snapshot in time and space.

3.1.2 Spatial Scaling of Fungi

Fungi play a major role in biogeochemistry, shaping plant community structure, and ecosystem function (Berendsen, *et al.*, 2012). Fungi have a wide range of ecological relationships, including parasitism, mutualism, saprophytism and commensalism, and interact with plants, insects, animals and other microbes. Previously, it was believed that fungal distribution was not limited by dispersal, unlike macro-organisms, but was mainly driven by environmental factors and host distribution (Berkeley, 1863; Raper, *et al.*, 1958). This assumption remained until recent advances in molecular ecology (Taylor, *et al.*, 2006). It is now believed that even though some fungi have far-reaching dispersal patterns, many face the same dispersal barriers as larger organisms, including mountains and oceans, anthropogenic pressures, ecological factors and historical events (James, *et al.*, 1999).

The separation of fungi over distance may be comparable to the separation of macro-organisms over distance, for example when habitats are fragmented by geological barriers, including oceans and mountains. Peay, *et al.* (2007), investigated fragmentation of ectomycorrhizal (EcM) communities between tree islands, 10 m² – 10 000 m² apart, and reported a species-area slope of 0.20 - 0.23, which is similar to species area relationships of macro-organisms. Green, *et al.* (2004), found predictable aggregated spatial patterns in Ascomycota fungi, using pairwise samples between 1m – 100km apart. In this study, geographical distance was a weak but significant influence on Ascomycota community structure, within land systems ($r = 0.04 - 0.16$) and across the Sturt National Park in Australia ($z = 0.074$), whereas land use type had no influence. Lilleskov, *et al.* (2004), found that EcM fungi display large similarity at distances < 2.6 m ($r = 0.02$, $p = 0.34$), however, communities decreased in similarity at greater distances, up to 300 m ($r = 0.22$, $p = 0.001^*$).

Within metre distances, Edman and Jonsson (2001) found there was no dispersal barrier to wood-decaying fungi on logs, in a forest in Sweden. However, at the microscopic scale, some fungi had variable distribution patterns, including EcM fungi on root tips (Tedersoo, *et al.*, 2003). There may be many factors at play in determining fungal spatial structure, which vary from landscape to the fine scale. A number of studies have also analysed the environmental factors which may cause spatial variability in fungal distribution. Tedersoo, *et al.* (2012) investigated the underlying processes determining EcM fungal community structure in a meta-analysis of 69 studies covering all EcM biomes, and found the major influences to be host plant family, temperature, and moisture. Similarly, Peay, *et al.* (2013) found beta-diversity of tropical forest soil fungal communities to be positively correlated with plant community, and environmental factors. Soil properties have also been found to influence fungal community structure. Cox, *et al.* (2010) found N to be the primary determinant of EcM composition in conifer forests.

3.1.3 Temporal Scaling of Fungi

Fungal community structure is also under the influence of time, which is an important variable influencing ecosystem structure and function. A number of studies have shown fungal community structure changes over time, including in EcM and Ascomycota fungi (Koide, *et al.*, 2007; Pereira e Silva, *et al.*, 2012). Abiotic factors may affect fungal temporal patterns, including temperature, fire, and rainfall (Hawes, *et al.*, 2006; Stendell, *et al.*, 1999). Biotic factors may also affect turnover of fungal communities, including plant physiology, biogeochemistry, succession, colonisation, and extinction (Hanski and Gyllenberg, 1993). Pereira e Silva, *et al.* (2012), found that total fungal (ITS region) community structure was relatively stable in Norwegian agricultural soils over three years, and while no soil parameters correlated significantly with fungal temporal β -diversity, 6.9 % of temporal fungal variability was explained by soil parameters. Amend, *et al.* (2009) found greater EcM diversity in forest stands over 50 years old. Neither of these studies could determine the source of variation between time points, therefore unknown factors were responsible for species turnover. Fungal communities have also been found to vary seasonally or over shorter time scales. Davey, *et al.* (2012), found across five time points in the phyllosphere of bryophytes, collection date was a significant component determining general fungal community structure. However, precipitation, soil and

air temperature were also significant factors, and samples were taken over less than a year, making it impossible to say whether turnover was due to time or season (Davey, *et al.*, 2012).

3.1.4 Spatial Scaling of AMF

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs, only surviving when associated with host plant roots, which limits their dispersal by hyphal growth. Recent evidence has shown that AMF are spatially structured at both local and landscape levels, using AMF specific 18S rRNA (Davison, *et al.*, 2012; Mummey and Rillig, 2008; van der Gast, *et al.*, 2011a). Opik, *et al.* (2013) sampled AMF from different biomes in every continent, and found global communities were separated by biogeographic history and environmental conditions. At a regional scale (250 km), van der Gast, *et al.* (2011a) discovered that AMF community composition was affected by distance. Davison, *et al.* (2012) also found significant differences in AMF communities in 10 x 10 m plots 30 m apart. Mummey and Rillig (2008), even found spatial autocorrelation in AMF ribotypes at distances < 1 m, and community patchiness at distances < 50 cm.

While some of the spatial variation in AM fungal community composition may be due to geographic distance, variation may also be due to the influence of underlying environmental factors, including pH, P, and rainfall (An, *et al.*, 2008; Hazard, *et al.*, 2013; van der Gast, *et al.*, 2011a). On a global scale across 111 published studies of natural habitats, Kivlin, *et al.* (2011) found that plant community, soil temperature and moisture, and geographic distance, influenced AMF community structure. However, a large proportion of variability was not determined, and the influence of soil nutrients were not included in the analysis. A number of studies have focused on soil properties. For example van der Gast, *et al.* (2011a), found that the most important factor determining spatial structure of AMF was environmental heterogeneity, and furthermore farming practice was linked to AMF diversity and turnover, which were higher in organic than conventional farms at distances <250 km. Hazard, *et al.* (2013) analysed the underlying environmental factors controlling AMF associated with *Lolium perenne* host plants across Ireland, and found community composition was influenced by rainfall, and pH.

3.1.5 Temporal Scaling of AMF

Temporal variation in AMF communities was previously observed using spore counts (Merryweather and Fitter, 1998). However spore morphologies are challenging to identify to species or strain level, which means genetic diversity is underestimated, and the presence of spores in the soil does not necessarily mean nearby plants have been colonised. Recent studies, using molecular methods, have also shown that AMF community structure is temporally variable in plant roots, including cheat grass (Busby, *et al.*, 2011), maize (Tian, *et al.*, 2011), and Mediterranean shrub land (Sanchez-Castro, *et al.*, 2012). There is evidence that AMF turnover between seasons. Busby, *et al.* (2011) found AMF colonisation in cheatgrass was highest in the late growth season during flowering, and dramatically lower in freezing soil temperatures. Tian, *et al.* (2011) found AMF colonisation of maize peaked in early reproductive growth, and the community was mainly affected by depth and growth stage, rather than soil management. Dumbrell, *et al.* (2011) noted an AMF community shift from summer to winter in a temperate grassland, and diversity was negatively correlated to temperature. Whereas, Davison, *et al.* (2012) did not find significant AMF turnover over a growing season in a natural forest soil. However, many of these studies only sampled during one year, which makes it impossible to conclude whether the community shift was seasonal, or if communities continually change over time in an unpredictable manner. There is evidence that AMF composition changes over longer periods of time. Husband, *et al.* (2002) found the AMF communities of *Tetragastris panamensis* seedlings significantly changed over three years, and seedlings of different ages consisted of significantly different fungal communities, which shows that turnover may be influenced by time and plant physiological factors, rather than season.

3.1.6 Commonness and rarity

In most environments, a few species dominate the community, some are less common, but most are rare (Magurran, 2004). A number of models have been developed to describe these species distributions, including log normal (Preston, 1960), geometric series (Motomura, 1932), and log series (Fisher, 1943). Other models, have been designed to identify species common to certain ecotypes, for example diagnostic species to help identify a type of vegetation (Chytry, *et al.*,

2002), or indicator species to monitor environmental changes for conservation purposes (Dufrene and Legendre, 1997).

3.1.7 The core-satellite species hypothesis

The core-satellite species (CSS) hypothesis, proposed that communities can be divided into 'core' species, which are locally abundant and regionally persistent, or 'satellite' species, which are regionally and locally rare (Hanski, 1982a; Magurran, 2007). Magurran and Henderson (2003) hypothesised that core species are well adapted to surroundings, whereas satellite species are under limitations of dispersal. Further development of CSS has tried to link community structure with ecosystem function to explain the difference between common and rare species, for example by combining CSS with Grime's dominant, subordinate, transient (GST) model (Gibson, *et al.*, 1999; Grime, 1998). Distribution of species in a meta-community can be widely variable, due to different mechanisms of dispersal and ecological function. This is further exacerbated as the majority of species are rare, and sampling error is greatest in rare species, making it difficult to generalise core and satellite organisation (Magurran, *et al.*, 2011). However, testing these models on more datasets could further refine the CSS hypothesis.

3.1.8 Core and satellite models

While the assumptions behind core and satellite species concepts are common to many studies, the models used to define 'core' and 'satellite' vary. Magurran and Henderson (2003) classified core species using a mean/variance ratio, removing log-series data as satellite, and fitting the rest into a log normal distribution. However, when data from this study was split into random and non-random, from the Poisson distribution, there were many anomalies of taxa with low persistence identified as core, and highly persistent taxa identified as satellite. It could be the model works better for macro-organisms, where population numbers are smaller, generation times are longer, and integer number counts are used to estimate populations, rather than relative abundance of sequence data, as used for microbial identity and frequency. However, Ulrich and Zalewski (2006) hypothesised that Magurran's Poisson model could miss patterns of dispersal and spatial distribution, including co-occurrence of species. Instead, some authors have used a temporal persistence threshold, by

classifying taxa in over 50 % or 75 % of time points as core (Magurran and Henderson, 2003; Unterseher, *et al.*, 2011). Monteils, *et al.* (2008) selected potential core gut bacteria as those found previously. Other authors have used abundance to divide communities (Siqueira, *et al.*, 2012; Unterseher, *et al.*, 2011). However, most studies have used rank abundance plots, which do not consider persistence, i.e. the proportion of samples they are detected in. Abundance may not be as relevant or reliable for microbial data, as many factors can alter abundance data away from the biological reality, including PCR bias, gene copy number, or sequencing of spores, which can massively overestimate certain taxa, and skew data, leaving other taxa underestimated.

3.1.9 Core and satellite examples in macro-organisms and microorganisms

A number of studies have shown differing ecological function between core and satellite communities. Magurran and Henderson (2003) found, in an estuary, that 'core' fish were species adapted to estuarine conditions, whereas satellite species were more adapted to other environments. Similarly, Ulrich and Zalewski (2006) studied beetles in a lake in Poland, and inferred that core beetle distribution was influenced by niches, whereas satellite beetles were randomly dispersed. Siqueira *et al* (2012), investigated macro-invertebrates in four streams, and found that environment and space could explain more variance in the core species, whereas satellite species were less influenced by environment, and even less so by the spatial component.

A number of studies have tried to identify the core microbiome in humans and animals, to identify deviations from the 'healthy' core that can lead to physiological differences. For example, Turnbaugh, *et al.* (2009) compared gut microbiota between obese and lean twins, and identified distinct core genes and phylum level changes separating the two groups. Turnbaugh also demonstrated that a core microbiome can be created from functional genes, rather than species similarity. Monteils, *et al.* (2008) analysed the gut bacteria of rabbits, and hypothesized that the most common species would respond differently to environmental and spatial factors, but provided no direct evidence.

3.1.10 Core and satellite fungi

The CSS hypothesis has to date only been applied to fungal diversity by one author (Unterseher, *et al.*, 2011; Unterseher, *et al.*, 2012). However, Unterseher used rank-abundance curves, which as previously discussed, may not be suitable for microbial community analysis, as it focuses only on abundance and does not incorporate persistence across samples, spatially or temporally. The benefit of using the 75 % threshold on persistence from the distribution abundance relationship (DAR), is if the DAR is a positive significant relationship, as is often found, then it takes into account abundance as well as persistence.

3.1.11 Aims

The aim of this study was to compare the extent to which the dispersal of total rhizosphere fungi and AMF are limited by geographic space, time, or environmental factors. Furthermore, the factors determining distributions of core and satellite total fungi and AMF were elucidated. A commercial *Miscanthus giganteus* field was investigated as a model field system.

3.2 Methods

3.2.1 Study site and root sampling

The study site was a *Miscanthus giganteus* biomass cropping field, established in 2006 near Lincoln, Lincolnshire, UK. Prior to *M. giganteus*, the field had a rotation of oilseed rape for 1 year, and wheat for 3 years. The crop was planted at a density of 10 000 rhizomes ha⁻¹. There was no N fertilization applied during or subsequent to establishment (Drewer *et al.*, 2012). The soil was a dense, compacted sandy loam with 53% sand, 32% silt and 15% clay, a BD of 1.51 ± 0.02 g cm⁻³ (n = 10), chemical properties of which are shown in **Figure 3.1**. Samples were collected near the beginning of the growth season (June), at the peak growth season (August), and at the end of the growth season (October). Soil samples were collected on 6th October 2010, and 21st June, 9th August, and 10th October 2011. Four replicates, 1 m apart from the central point, were taken from eight intervals, 20 m apart, and 25 m parallel to the field edge. Soil cores (0 - 15 cm) were collected, stored at 5 °C, and processed within a week. Roots

were removed from the soil using tweezers. Healthy white roots were separated from old and senescent roots, which were blackened, and washed in deionized water. The roots were cut to 1cm lengths, thoroughly mixed, and stored at either -20 °C, or in 20 % (v/v) ethanol at 4 °C, for molecular and staining analyses respectively.

3.2.2 Soil Analysis

The soil was a sandy loam with 15 % clay, 53 % sand and 32 % silt (Case, *et al.*, 2012). The following soil properties were measured in each sample. pH was measured with a Russell model RL 150 pH meter, in a solution ratio of 1:5 with deionized water. Nitrate and ammonium were extracted from soil with 0.5 M K₂SO₄ (saturated solution) and quantified using a Foss FIAStar 5000 flow injection analyser. Soil was oven dried at 60 °C prior to further analysis. % C and % N were measured by combustion using a Leco CN2000 analyser. Available P was extracted from dry soil with 0.5M sodium hydrogen carbonate (pH 5.8). Available K and Mg were extracted from dry soil with 1M ammonium nitrate. P, K, and Mg were quantified using a Jobin Yvon Ultima 2 ICP-OES, inductively coupled plasma optical emission spectrometer.

3.2.3 Mycorrhizal staining and colonisation

Roots were stained to visualize AM fungi and to calculate root length colonization by AMF (Grace and Stribley, 1991). Roots were thoroughly washed, covered in 10% KOH, and incubated at 90 °C for 1.5 h. Samples were then washed with deionised water and covered in 2 % HCL for 5 min. Roots were immersed in aniline blue stain (0.05 % in 70 % glycerol) and incubated at 90 °C for 10 min. Stained roots were covered in acidified glycerol (50 % glycerol, 0.02 % HCL). Root length colonisation was estimated in each sample, using 10 fields of view from 10 roots, using methods previously described (Brundrett, *et al.*, 1996).

3.2.4 Molecular methods

3.2.4.1 DNA extraction

DNA was extracted from 200 mg of washed and dried roots, using a QIAGEN FastDNA SPIN Kit for Soil, according to manufacturer's instructions, and stored

at -20 °C.

3.2.4.2 Total fungal PCR

For total fungal analysis, internal transcribed spacer (ITS) DNA was amplified using the general fungal primer set ITS1F (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White, *et al.*, 1990), fluorescently labeled with 6-FAM. ITS PCR cycle conditions were as follows: 95 °C 3 min, 30 cycles of 95 °C 3 min, 55 °C 1 min, 72 °C 1 min, then 72 °C 10 min, 12 °C.

3.2.4.3 AMF PCR

For AMF analysis, 18S rDNA fragments were amplified using the AMF specific primers AML1 (5-ATCAACTTTCGATGGTAGGTAGGATAGA-3) and AML2 (5-GAACCCAAA CACTTTGGTTTCC-3) (Lee, *et al.*, 2008). AMF PCR cycle conditions were as follows: 94 °C 5 min, 94 °C 1 min, 40 cycles of 57 °C 1 min, 72 °C 1 min, then 72 °C 10 min, 12 °C. PCR products were purified using a QIAquick PCR purification kit.

3.2.4.4 Sequencing

AMF transcripts were sequenced, to identify the presence of AMF species, to check for any non-AMF sequence amplification, and to choose restriction enzymes for T-RFLP. Two clone libraries were created using pooled DNA from October 2010 and from June 2011. 18S rDNA was amplified using the method described above, with unlabeled AML1 and AML2 primers. PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN House, Sussex, UK), following the manufacturer's protocol. Cloning was carried out using the QIAGEN PCR cloning plus kit (QIAGEN House, Sussex, UK), and TempliPhi Amplification kit (GE Healthcare UK Ltd.), following manufacturer's protocol. Products were analysed on an Applied Biosystems 3130X 1 automate capillary sequencer. Sequences were edited and analysed using DNASTAR 9 (Lasenger, Madison, USA).

3.2.4.5 Fragment Length Analysis

Terminal restriction fragment length polymorphism (T-RFLP) was performed on PCR products, amplified as described above, with 6-FAM fluorescently labeled primers, to assess diversity of arbuscular mycorrhizal fungi and total fungi. Restriction enzymes were chosen by fragmenting clone library sequences *in silico* with all restriction enzymes listed on bioperl Restriction Enzyme Mapping Application (REMA) <http://bioperl.macaulay.ac.uk>. The restriction enzyme which generated the highest TRF richness from the clone library, *Ase1*, was chosen for AMF 18S rDNA, and a commonly used restriction enzyme for ITS rDNA (Hilton, *et al.*, 2013), *MspI*, was used for total fungi (New England Biolabs, UK). T-RFLP reactions contained 300 ng of PCR product, 10 % buffer, 2 units of restriction enzyme, made up to 30 µl with pure water. Samples were incubated for 4 h at 37 °C, followed by 20 min at 65 °C to denature enzymes. Mixtures of 1 µl digestion product, 9.85 µl formamide, and GeneScan™ Size Standard (LIZ® 1200 for 18S rDNA, and LIZ® 600 for ITS DNA) were denatured for 5 min at 95 °C, cooled on ice, and analysed on an Applied Biosystems 3130X1 automated capillary sequencer. TRF profiles were analysed using GeneMarker v1.8 (SoftGenetics, Stat College, USA). TRFs above 1 % relative abundance of that sample, and between 100-550bp for 18S rDNA, and 100-750bp for ITS DNA, were included in the analysis. The relative abundance of each TRF was used to create a matrix for diversity analysis. Each AM TRF was identified, by matching fragment lengths to *in silico* digestion products of clone library sequences.

3.2.5 Data Analysis and Statistics

3.2.5.1 Core and Satellite taxa

Two models were applied to determine which taxa were the 'Core' species, with high persistence (frequency of detection) and abundance (relative mean proportion of sequences), and which were 'Satellite' species, with low persistence and abundance; selection of a persistence threshold (Siqueira, *et al.*, 2012; Unterseher, *et al.*, 2012), and using Magurran and Henderson's random and non-random Poisson distribution (Magurran, 2004; Magurran and Henderson, 2003; van der Gast, *et al.*, 2011b). However, the persistence threshold was better suited to the data, with fewer anomalies, therefore only the persistence threshold analysis is presented.

3.2.5.2 Persistence threshold method for core and satellite taxa

Persistence was exponentially correlated with mean abundance (mean *Exponential R*², AMF = 0.874, **Figure 3.10**, Fungi = 0.594, **Figure 3.12**). It was clear that highly persistent taxa were also highly abundant, whereas less persistent taxa were far less abundant. The point of inflection in the exponential curve was around 75 % in both AMF (**Figure 3.12**) and total fungi (**Figure 3.10**). Therefore, taxa found in > 75 % samples, were classified as core taxa, anything found in < 75 % of samples, was classified as satellite.

3.2.5.3 Diversity and temporal scaling of core and satellite taxa

To assess α -diversity and β -diversity measures of total fungi and AMF community structure, Bray Curtis dissimilarity matrices were made. For α -diversity and β -diversity measures of core and satellite taxa, a Sorensen similarity distance matrix was created. Similarity matrices were then used for; SIMPER (similarity percentage) analysis, ANOSIM (analysis of similarity), and to create an NMDS (non- metric dimensional scaling) plot overlayed with clustering at 20, 40, 60, and 80 % similarity, using Primer-6 software (Primer-E, Lutton, UK).

3.2.5.4 Distance Decay of total fungal and AMF communities

Distance decay relationships were modeled using the power law equation $S = cD^d$, where S = pair-wise similarity for any two samples using Bray Curtis (S_{BC}) quantitative indices of similarity, c = constant, D = distance between pair-wise samples, d = rate of decay in similarity (Green, *et al.*, 2004).

3.2.5.5 Influence of geographic distance and environmental dissimilarity on fungal and AMF community structure

All statistical analysis was performed using R package vegan (Oksanen, *et al.*, 2011). To measure correlation between geographic distance and community similarity, Mantel tests were carried out using the equation $r(SD)$. To determine how much of the variability in species composition was explained by geographic distance, and if residual variability was spatially structured, partial Mantel tests were carried out, according to the equation: $r(SD.x)$ (Green, *et al.*, 2004)

whereby x = soil property. Conversely, to determine how much of the variability in species composition was explained by environmental variability, and if residual variability was spatially structured, partial Mantel tests were carried out according to the equation: $r(Sx.D)$, (Green, *et al.*, 2004) whereby x = soil property. The partial Mantel test measures partial regression on the three distance matrices: species dissimilarity, geographic dissimilarity, and environmental dissimilarity (Legendre and Fortin, 1989). To determine whether communities were stable across time, the variability between species composition (Bray Curtis similarity matrixes of TRF relative abundance) at each time point, was analysed using an Analysis of Similarity (ANOSIM). Pearson product moment correlation was used to find correlations between soil properties.

3.2.5.6 Correlations between environmental variables, colonisation and TRF richness

Pearson product-moment correlation tests were performed between AMF percent root length colonisation and each environmental variable. Pearson product-moment correlation tests were also performed between each environmental variable and total fungal and AMF TRF richness and individual TRFs.

3.3 Results

3.3.1 Soil properties

The soil was a sandy loam with mean (\pm S.D.) properties as follows: pH 6.13 (± 0.3), 1.84 (± 1.9) % C, 0.13 (± 0.09) % N, 2.02 (± 1.7) NO₃ (mg/kg), 3.10 (± 1.6) NH₄ (mg/kg), 109.4 (± 35) K (mg/kg), 200.3 (± 90) Mg (mg/kg), and 31.4 (± 8.7) P (mg/kg). Soil properties from each sampling time are listed in **Table 3.1**. The soil properties were relatively stable over time.

Table 3.1. Mean (\pm S.D.) soil characteristics from the Brattleby field site 6th October 2010, 21st June 2011, 9th August 2011, and 10th October 2011, from all sampling locations.

Time	pH	%C	%N	NO ₃ (mg/kg)	NH ₄ (mg/kg)	K (mg/kg)	Mg (mg/kg)	P (mg/kg)
Oct-10	6.25 (0.1)	1.86 (0.4)	0.13 (0.03)	4.06 (2.2)	1.34 (0.5)	114.2 (52)	195.5 (59)	21.5 (4.6)
Jun-11	6.09 (0.3)	1.88 (0.3)	0.14 (0.03)	1.08 (0.5)	5.07 (1.5)	115.0 (30)	211.7 (81)	31.3 (4.8)
Aug-11	5.93 (0.4)	1.82 (0.3)	0.13 (0.13)	1.53 (0.6)	2.88 (0.4)	103.0 (25)	211.2 (127)	34.8 (7.5)
Oct-11	6.24 (0.3)	1.78 (0.2)	0.13 (0.13)	1.43 (0.4)	3.13 (0.5)	105.3 (27)	182.9 (79)	38.2 (7.2)
Mean	6.13 (0.3)	1.84 (1.9)	0.13 (0.09)	2.02 (1.7)	3.10 (1.6)	109.4 (35)	200.3 (90)	31.4 (8.7)

3.3.2 Total rhizosphere fungi

3.3.2.1 Temporal scaling of total fungi

There were 136 total fungal TRFs, with a mean number per sample of 18.6 taxa in Oct-10, 16.9 taxa in Jun-11, 17.9 taxa in Aug-11, and 19.8 taxa in Oct-11 (**Figure 3.1.**). The only significant difference in taxa richness, between sampling times, was between Jun-11 and Oct-11 (TTEST, $P = 0.01$).

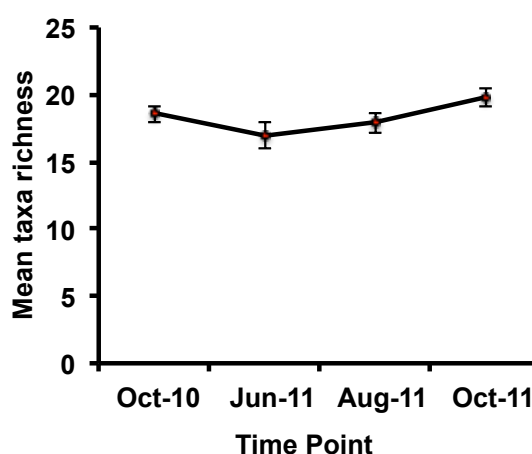


Figure 3.1. Mean total fungal taxa (TRF) richness per sample, across time. Error bars represent \pm standard error of the mean.

Total fungal communities significantly changed between all time points, except between Aug-11 and Oct-11 (ANOSIM $r^2 = 0.02$, $P = 0.12$) (**Table 3.2.**). The temporal shift in fungal composition was clear on a non-metric multidimensional scaling (NMDS) plot (**Figure 3.2.**), with clustering of time points and small shifts between each time point, showing communities were more similar within, than between, time points.

Table 3.2. Average dissimilarity in communities of total fungi. r^2 values are displayed in the top right triangle, and p values in the bottom left triangle. Where, * represents a significant difference at the P level indicated.

	Oct-10	Jun-11	Aug-11	Oct-11
Oct-10	x	0.345	0.401	0.346
Jun-11	0.001*	x	0.086	0.149
Aug-11	0.001*	0.002*	x	0.02
Oct-11	0.001*	0.001*	0.12	x

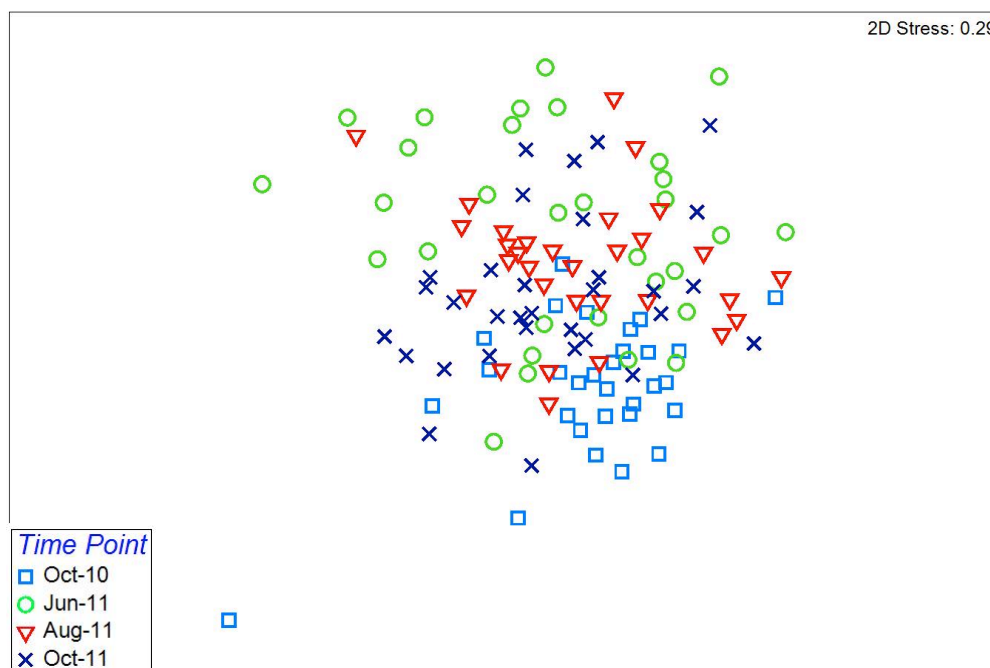


Figure 3.2. Non-metric multidimensional scaling of total fungal community structure, using Bray Curtis similarity. Each point represents the community of a sample, differentially coloured by time point (see key).

3.3.2.2 Temporal persistence and abundance of total fungi

The majority of fungal taxa had low persistence and relative abundance, with an overall mean of 13.3 % (S.E.M. \pm 0.88 %) and 0.6 % (S.E.M. \pm 0.1 %) respectively. The abundance and persistence of each fungal taxon changed over time (**Table 3.3**). One hundred taxa were consistently < 25 % persistent, two taxa consistently > 75 % persistent, and the remaining taxa were more variable over time. In at least one time point, ten taxa were > 75 % persistent (coloured purple **Table 3.3**), thirteen taxa > 50 % persistence (coloured red **Table 3.3**), and thirty-six taxa > 25 % persistence (coloured yellow **Table 3.3**).

Persistence over time was highly variable, many peaked or troughed at certain time points, and others rose or fell over time.

Table 3.3 Mean relative abundance of total fungal TRFs, overlaid with persistence.

TRF	Oct-10	Jun-11	Aug-11	Oct-11	TRF	Oct-10	Jun-11	Aug-11	Oct-11	<div><div></div><div></div><div></div></div>	Colour	Persistence		
100	1.1	0.7	1.4	0.3	178	0.0	0.0	0.1	0.6					
102	1.8	1.4	1.3	1.2	180	0.0	0.2	0.1	0.1					
104	0.6	1.3	0.7	0.4	181	0.4	0.4	0.4	0.3					
105	9.2	3.7	3.9	4.3	183	0.1	2.0	0.1	0.3			25 -50 %		
106	0.0	0.0	0.1	0.3	185	0.0	0.0	0.0	0.0			0 - 25 %		
108	0.0	0.0	0.7	0.0	186	0.7	1.1	1.8	2.2					
111	0.1	0.1	1.7	0.4	188	0.0	0.1	0.1	0.0					
117	0.0	0.1	0.0	0.1	190	0.1	0.1	0.3	0.6	TRF	Oct-10	Jun-11	Aug-11	Oct-11
119	0.0	0.7	0.1	0.0	192	0.2	0.3	0.0	0.1	330	0.0	0.1	0.2	0.0
122	0.0	0.1	0.0	1.3	196	0.0	0.2	0.0	0.1	332	0.0	0.1	0.0	0.0
123	0.6	2.3	0.3	0.1	198	1.9	0.4	0.7	0.7	333	0.0	0.4	0.1	0.1
125	8.5	2.7	2.6	2.3	200	0.1	0.1	0.2	0.6	334	0.1	0.1	0.0	0.1
127	0.2	0.0	0.1	0.3	201	0.1	0.2	0.5	0.6	336	0.1	0.0	0.2	0.0
129	0.1	0.7	0.7	0.1	202	0.4	0.0	0.0	0.0	337	0.1	0.0	0.0	0.0
129	0.4	0.9	0.0	0.7	205	0.0	0.0	0.0	0.0	338	0.2	0.4	0.4	0.1
130	0.8	0.6	1.4	0.9	206	0.1	0.0	0.2	0.5	358	0.0	0.0	0.0	0.0
132	1.5	1.5	1.8	0.7	213	0.0	0.0	0.2	0.1	361	0.2	0.3	0.3	0.0
133	0.0	0.0	0.0	0.0	215	0.0	0.0	0.0	0.3	372	0.1	0.0	0.0	0.0
133	0.3	1.0	0.2	0.5	216	0.0	0.0	0.0	0.2	374	0.2	0.0	0.1	0.0
135	0.1	0.2	0.1	0.1	220	0.0	0.0	0.4	0.1	395	0.0	0.1	0.0	0.0
136	0.0	0.4	0.3	0.0	221	0.0	0.1	0.0	0.0	418	0.0	0.4	0.3	0.1
137	2.0	0.0	0.0	0.0	223	0.2	0.2	0.0	0.2	419	0.8	0.4	0.1	0.0
140	14.6	11.8	15.9	16.8	224	0.5	0.3	0.4	0.3	421	0.9	0.9	0.8	1.5
141	0.6	1.2	0.2	0.6	226	1.8	15.1	14.9	9.7	422	0.1	1.0	1.3	1.8
143	0.3	0.1	0.0	0.2	228	0.1	0.1	0.0	0.3	423	0.8	1.6	2.0	2.4
144	1.1	1.3	0.2	0.2	239	0.2	0.2	0.1	0.5	428	0.2	0.1	0.0	0.3
145	4.4	2.0	1.9	1.8	240	0.0	0.0	0.2	0.0	431	0.6	1.7	0.3	0.5
146	0.0	0.1	0.9	0.1	242	0.1	0.0	0.1	0.4	433	0.7	0.2	0.0	0.2
147	0.1	0.9	0.0	0.2	249	2.9	0.0	1.8	0.2	440	3.4	1.4	1.1	1.1
148	0.0	0.0	0.3	0.0	255	0.3	0.0	0.0	0.1	442	2.9	0.1	0.1	0.5
150	0.1	0.0	0.2	0.0	258	0.0	0.0	0.0	0.0	452	0.0	0.0	0.0	0.4
152	0.0	1.1	0.0	0.5	260	0.0	0.1	0.2	0.1	459	0.1	0.7	1.5	0.6
154	0.0	0.0	0.0	0.1	266	0.0	0.1	0.0	0.0	461	0.0	1.6	0.0	0.1
156	0.3	0.2	0.0	0.0	267	0.0	0.0	0.2	0.0	463	1.3	2.9	0.9	1.3
158	0.9	0.3	0.0	0.6	268	0.1	1.2	0.4	0.1	468	0.0	0.0	0.4	0.7
160	2.4	3.5	6.1	5.9	269	0.2	0.1	0.2	0.1	469	0.6	1.0	0.7	1.2
161	2.5	1.1	0.8	1.3	270	0.0	0.0	0.0	0.2	476	1.3	0.3	0.4	0.6
162	0.7	1.3	0.3	1.2	271	0.1	0.0	0.2	0.0	484	0.1	0.3	0.1	0.3
164	0.0	0.0	0.0	0.3	274	0.0	1.5	0.1	0.0	494	2.5	0.8	1.1	1.3
165	0.3	0.1	0.3	0.6	291	0.0	0.0	0.0	0.1	496	0.2	0.6	1.1	1.0
167	0.0	0.0	0.1	0.2	296	0.0	0.4	0.0	0.0	498	0.3	0.2	0.1	0.2
167	0.0	0.0	0.0	0.2	302	0.0	0.0	0.0	0.3	502	0.2	0.2	0.0	0.0
168	0.5	0.1	0.0	0.2	319	0.0	0.0	0.2	0.0	503	0.1	0.0	0.0	0.2
169	0.0	0.0	0.0	0.1	320	0.0	0.1	0.1	0.1	508	0.1	0.0	0.2	0.0
172	0.0	0.1	0.0	0.0	321	0.0	0.0	0.0	0.1	511	0.0	1.2	0.4	0.4
173	0.0	0.4	1.1	0.0	323	0.0	0.0	0.0	0.1	514	2.4	0.2	0.0	0.2
175	0.1	0.0	0.0	0.0	326	0.0	0.1	0.0	0.0	517	1.2	0.5	0.5	0.0
176	0.0	0.0	0.1	0.0	327	0.1	0.0	0.0	0.9	519	0.0	0.0	0.0	0.5

The fungal taxon accounting for the greatest SIMPER variability was TRF 226, which contributed to 10.3 % of dissimilarity between time points. The other taxa contributing largest variability (%) between time points include; TRF 140 (8.5 %),

TRF 105 (4.5 %), TRF 125 (4.0 %), and TRF160 (3.5 %).

3.2.2.3 Distance decay of total fungal communities across a field

There was a significant distance decay relationship, of total fungal community structure, in June 2011 ($S_{BC} = 0.40D^{-0.094}$, $r^2 = 0.091$, $P = <0.0001^*$). However, there was no distance decay in October 2010 ($S_{BC} = 0.30D^{-0.001}$, $r^2 = <0.0001$, $P = 0.95$), August 2011 ($S_{BC} = 0.35D^{-0.016}$, $r^2 = 0.003$, $P = 0.30$), or October 2011 ($S_{BC} = 0.38D^{-0.024}$, $r^2 = 0.013$, $P = 0.28$) (**Figure 3.3**).

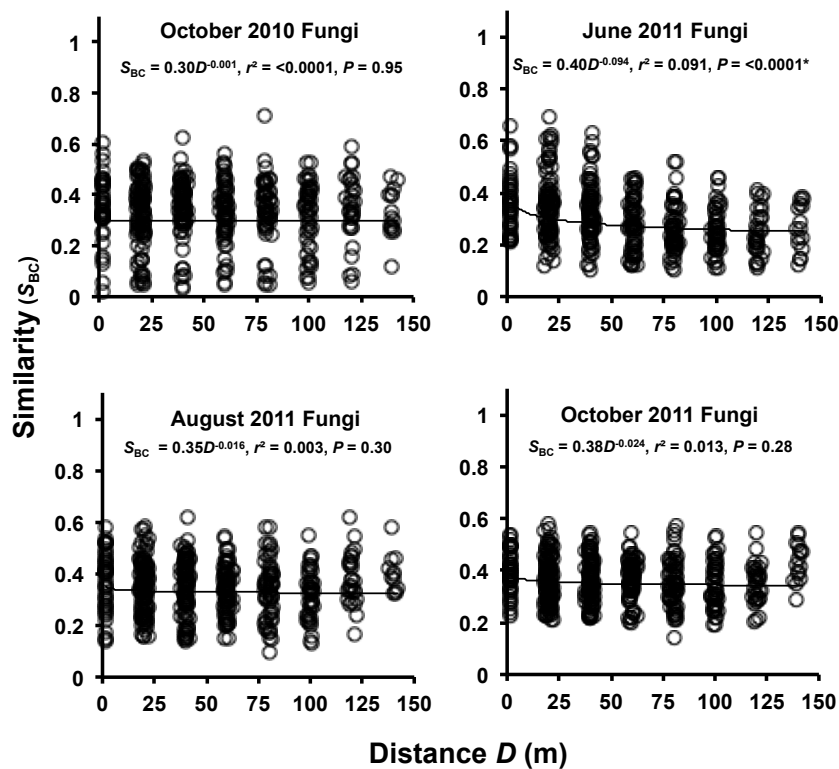


Figure 3.3 Pairwise total fungal community similarity, at increasing geographic distance, with Bray Curtis indices of similarity. In each instance, given is the distance-decay relationship power law equation, $S = cD^d$. * = $P < 0.01$. Mantel and partial Mantel summary statistics are given in **Table 3.4**.

3.3.2.4 Influence of geographic distance and environmental distance on total fungal community structure

Accumulating all time points, total fungal community structure was not significantly influenced by either environmental (ANOSIM $r = 0.071$, $P = 0.115$) or geographical variation (ANOSIM $r = 0.002$, $P = 0.504$) (**Table 3.4**).

Table 3.4 Summary statistics of Mantel tests across all time points, correlating community structure (Bray Curtis similarity distance matrix of TRF relative abundances) with Geo (Euclidian geographic dissimilarity matrix), and Env (Euclidian environmental dissimilarity matrix of pH, %N, %C, NO₃, NH₄, P, K, Mg, and C:N). Geo(Env) represents the partial Mantel test between geographical distance and community structure, controlling for environmental variability. Env(Geo) represents the partial Mantel test between environmental distance and community structure, controlling for Geographical variability. * = $P < 0.01$.

Factor	Fungi	
	<i>r</i>	<i>p</i>
Geo	0.032	0.135
Env	0.078	0.099
Geo(Env)	0.002	0.504
Env(Geo)	0.071	0.115

3.3.2.5 Influence of environmental factors on total fungal community structure

In June 2011, fungal community structure was affected by geographical distance ($r^2 = 0.226$, $P = 0.001^*$), and no soil properties. In August 2010, only the residual variability of NO₃ ($r^2 = 0.288$, $P = 0.009^*$) significantly correlated with community composition. In October 2010 and October 2011, total fungal composition was not significantly influenced by geographic distance or environmental factors (Table 3.5).

Table 3.5 Distance decay statistics for total fungal community structures. Summary of Mantel and Partial Mantel tests, using Bray Curtis indices of similarity, whereby * $p = < 0.01$ significance.

Parameter Control For		Oct-10		Jun-11		Aug-11		Oct-11	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Distance		0.004	0.450	0.266	0.001 *	0.041	0.284	-0.019	0.604
Distance	Env	0.018	0.389	0.217	0.007 *	-0.1007	0.859	-0.060	0.777
Distance	pH	-0.007	0.502	0.253	0.001 *	0.004	0.432	-0.089	0.908
Distance	%C	-0.007	0.505	0.220	0.004 *	0.004	0.465	-0.041	0.678
Distance	%N	-0.042	0.627	0.178	0.022	0.001	0.479	-0.030	0.593
Distance	NO ₃	0.004	0.454	0.233	0.003 *	-0.059	0.741	-0.045	0.727
Distance	NH ₄	-0.003	0.467	0.273	0.001 *	0.029	0.322	-0.020	0.607
Distance	K	-0.001	0.476	0.265	0.001 *	0.045	0.261	-0.026	0.630
Distance	Mg	-0.044	0.640	0.210	0.004 *	-0.099	0.874	-0.059	0.763
Distance	P	0.028	0.375	0.271	0.001 *	0.047	0.269	-0.056	0.761
Env		-0.040	0.555	0.174	0.081	0.256	0.026	0.074	0.257
Env	Distance	-0.044	0.596	0.076	0.246	0.271	0.019	0.093	0.239
pH	Distance	0.048	0.249	0.017	0.389	0.259	0.017	0.224	0.020
%C	Distance	0.033	0.351	0.025	0.406	0.061	0.284	0.041	0.318
%N	Distance	0.090	0.233	0.085	0.244	0.049	0.302	0.023	0.389
NO ₃	Distance	-0.012	0.454	0.200	0.018	0.288	0.009 *	0.127	0.125
NH ₄	Distance	0.032	0.302	-0.082	0.780	0.092	0.173	0.027	0.377
K	Distance	-0.191	0.979	-0.032	0.587	-0.060	0.650	0.090	0.152
Mg	Distance	0.115	0.192	0.086	0.234	0.272	0.023	0.092	0.237
P	Distance	-0.043	0.620	-0.062	0.741	-0.024	0.530	0.157	0.070

3.3.2.6 Influence of environmental factors on total fungal taxa richness

There were no environmental variables which significantly correlated with total fungal taxa richness (**Table 3.6**).

Table 3.6 Summary of correlations between environmental variables with total fungal TRF richness. Whereby, r = Pearson product-moment correlation coefficient, and * p-value < 1 %.

Parameter	Oct-10		Jun-11		Aug-11		Oct-11	
	r	P	r	P	r	P	r	P
pH	0.091	0.632	-0.130	0.486	-0.404	0.024	-0.297	0.099
N	0.078	0.681	-0.123	0.509	-0.104	0.577	0.022	0.903
NO ₃	0.249	0.184	-0.194	0.295	-0.448	0.012	-0.066	0.721
NH ₄	-0.224	0.234	-0.154	0.407	0.078	0.676	-0.104	0.573
C	0.167	0.376	-0.151	0.417	-0.075	0.687	0.003	0.986
K	0.060	0.753	0.056	0.764	0.140	0.453	-0.080	0.664
Mg	0.016	0.932	-0.029	0.878	-0.357	0.049	-0.210	0.250
P	0.146	0.442	-0.061	0.744	0.254	0.169	0.041	0.823

3.3.3 Arbuscular mycorrhizal fungi

3.3.3.1 AMF colonisation in *Miscanthus* roots

Stained AMF coils, arbuscles, hyphae, and spores, were observed under a light microscope in all samples and all seasons, which confirmed their presence in *M. giganteus* roots (**Figure 3.4**) Root length colonisation (RLC) ranged from 17 – 80 %, and varied seasonally. Mean (\pm SEM) RLC was greatest in Jun-11, at 68.1 % (\pm 0.98), and Aug-11 RLC, at 61.4 % (\pm 1.30), which were significantly lower than Jun-11 (TTEST, P = 0.005*). Mean RLC of October 2010 and October 2011, was 34.9 % (\pm 1.83), and 37.4 % (\pm 1.44) respectively, which were both significantly lower (TTEST, P < 0.0001*) than summer RLC's (**Figure 3.5**). Additional unstained fungal structures were also observed using Melzer's reagent.

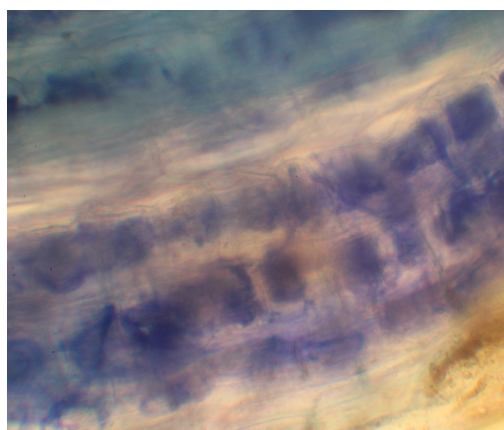


Figure 3.4 Arbuscules in root cortex cells, within *M. giganteus* field roots, collected 21st June 2011.

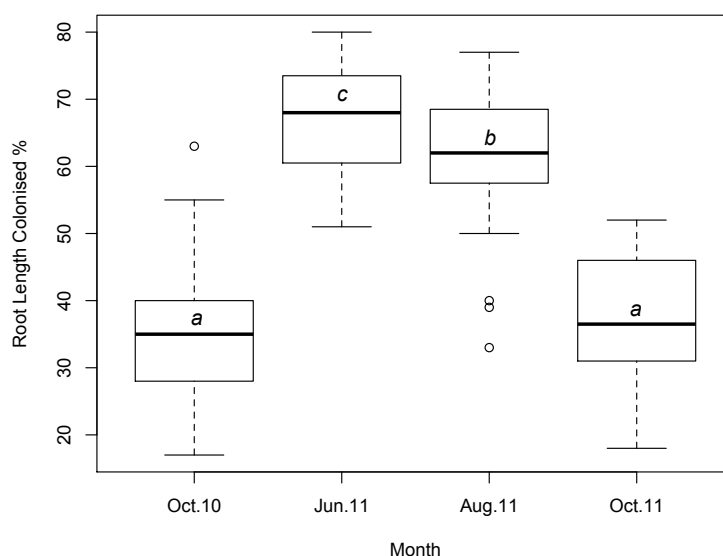


Figure 3.5 Boxplot of AMF root length colonisation; 6th October 2010, 21st June 2011, 9th August 2011, and 10th October 2011, where *a*, *b*, and *c* are significantly different means (TTEST).

3.3.3.2 AMF sequencing results

Out of 96 AMF clones, for Oct-10 and Jun-11, sequencing was successful for 87 and 79 clones, respectively (**Table 3.7**). At 97 % similarity, there were nine Operational Taxonomic Units (OTUs) in Oct-10, and fourteen OTUs in Jun-10. The abundance of each genotype varied between the time points, with greater alpha diversity in June (Shannon's Index Oct-10 = 1.83, and Jun-11 = 2.38).

Table 3.7 Oct-10 and Jun-11 AMF clone library summary, of uclust picked OTUs, and diversity indices.

Date	No. Clones	α -Diversity	Shannons	97%	uclust OTU's			
			Index		98%	99%	100%	
Oct-10	87	4.94	1.83	9	15	28	80	
Jun-11	79	2.53	2.38	14	17	27	73	

All clones were classified as Glomeromycota, which confirmed that AML1/2 TRFLP was specific to AMF, and there were no contaminating non-AMF sequences. A total of seven phylogenetically distinct groups were found, using BLAST (**Table 3.8**). The taxonomy included species from all four orders of the Glomeromycota. Three taxa dominated the clone library, *Glomus iranicum* (mean = 25.5 %), *Claroideoglomus lamellosum* (mean = 32.1 %) and *Paraglomus laccatum* (mean = 22.3 %), followed by rarer sequences of *Diversispora aurantia* (mean = 8.6 %), an uncultured *Ambispora* strain (mean = 8.5 %), *Paraglomus occultum* (mean = 2.5 %), and *Rhizophagus intraradices* (mean = 0.6 %). The number of clones of each taxa varied between Oct-10 and Jun-11.

Table 3.8 Phylogenetically distinct AMF clones, from Oct-10 and Jun-11

% Clones		Species	Order	Max Identity	Accession No.
Oct-10	Jun-11				
36.0	15.0	<i>Glomus iranicum</i>	Glomerales	98%	HM153424.1
27.9	36.3	<i>Claroideoglomus lamellosum</i>	Glomerales	98%	AJ276087.2
22.1	22.5	<i>Paraglomus laccatum</i>	Paraglomerales	99%	AM295493.1
5.8	11.3	<i>Diversispora aurantia</i>	Diversisporales	99%	AM713432.1
7.0	10.0	<i>Uncultured Ambispora</i>	Archaeosporales	99%	AJ276074.2
0.0	5.0	<i>Paraglomus occultum</i>	Paraglomerales	97%	NG_017179.1
1.2	0.0	<i>Rhizophagus intraradices</i>	Glomerales	96%	AM713432.1

3.3.3.3 Temporal Scaling of AMF

There were 21 AMF TRFs, with a mean number per sample of 11.0 taxa in Oct-10, 13.6 taxa in Jun-11, 13.5 taxa in Aug-11, and 13.7 taxa in Oct-11 (**Figure 3.6**). Oct-10 had significantly lower taxa richness than Jun-11 (TTEST, $P < 0.001$ *), Aug-11 (TTEST, $P < 0.001$ *), Oct-11 (TTEST, $P < 0.001$ *). There were no significant differences of taxa richness between the three time points in

2011. AMF communities significantly changed between all time points ($P < 0.01$), except between Jun-11 and Aug-11 (ANOSIM $r^2 = 0.01$, $P = 0.26$) (**Table 3.9**). The temporal shift in AMF composition was clear on an NMDS plot, with communities changing over time (**Figure 7**).

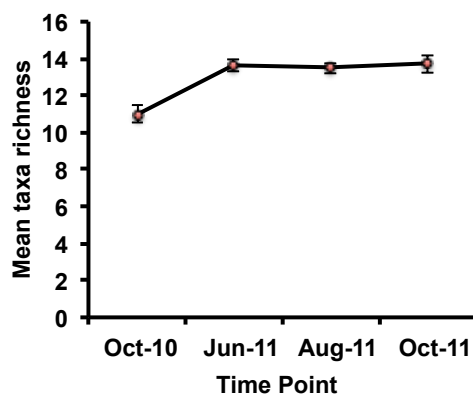


Figure 3.6 Mean taxa (TRF) richness per sample, across time for AMF. Error bars represent +/- standard error of the mean.

Table 3.9 Average dissimilarity in communities of AMF. r^2 values are displayed in the top right triangle, and p values in the bottom left triangle. Where, * represents a significant difference at the P level indicated.

	Oct-10	Jun-11	Aug-11	Oct-11
Oct-10	x	0.152	0.087	0.099
Jun-11	0.001*	x	0.01	0.178
Aug-11	0.004*	0.26	x	0.083
Oct-11	0.002*	0.001*	0.005*	x

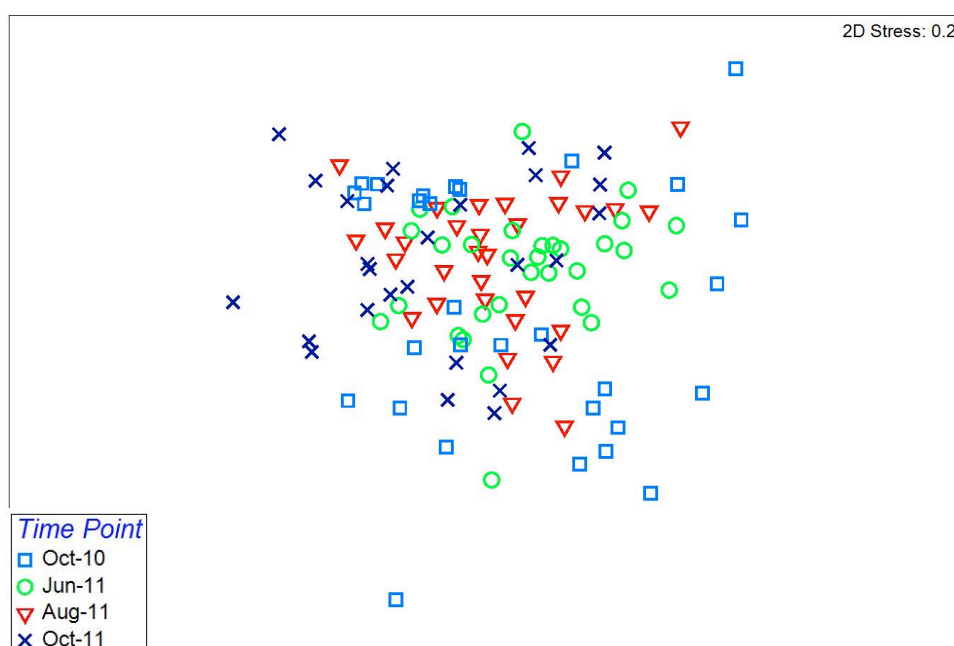


Figure 3.7 NMDS plot of microbial communities, using Bray Curtis similarity, of AMF community structure. Each point represents the community of a sample, differentially coloured by time point (see key).

3.3.3.4 Temporal persistence and abundance of AMF

The abundance and persistence of each AMF taxon changed over time (**Table 3.10**). Seven taxa remained consistently persistent over time, and the remaining fourteen peaked, dipped, rose, or fell in persistence and abundance over time (**Table 3.10**). The mean persistence and relative abundance of all taxa was 61.7 % (S.E.M. \pm 3.38 %), and 4.6 % (S.E.M. \pm 0.8 %), respectively. In at least one time point, fourteen taxa were > 75 % persistent, fifteen > 50 %, twenty > 25 %, and only one taxon remained < 25 %. The dominant taxon was a *Paraglomus laccatum* strain (TRF 369), with 96.6 % (S.E.M. \pm 3.4 %) persistence, and 20.4 % (S.E.M. \pm 2.7 %) relative abundance. Other dominant taxa included *Claroideoglossum lamellosum* (TRF 536), with 97.4 % (S.E.M. \pm 1.7 %) persistence and 12.0 % (S.E.M. \pm 1.4 %) relative abundance, and *Diversispora aurantia* (TRF 528) with 89.7 % (S.E.M. \pm 5.5 %) persistence and 10.8 % (S.E.M. \pm 1.8 %) relative abundance.

Table 3.10 Mean relative abundance of AMF TRFs, overlaid with percentage of persistence, and matched to the closest result from Blast (see **Table 3.8**).

TRF	Taxa best match	Mean Relative Abundance %				Colour	Persistence
		Oct-10	Jun-11	Aug-11	Oct-11		
369	<i>Paraglomus laccatum</i>	18.9	16.9	22.4	23.2		75-100 %
371	<i>Paraglomus laccatum</i>	2.3	2.0	3.0	2.7		50-75 %
372	<i>Paraglomus laccatum</i>	0.4	1.2	1.6	0.3		25-50 %
395	<i>Glomus iranicum</i>	0.8	1.3	2.6	11.4		
398	<i>Glomus iranicum</i>	3.8	5.5	5.2	2.3		
401	<i>Glomus iranicum</i>	1.4	0.6	0.3	0.8		
440	<i>Uncultured Ambiospora</i>	0.2	0.0	0.2	0.8		
444	<i>Uncultured Ambiospora</i>	0.1	0.1	0.4	1.1		
445	<i>Uncultured Ambiospora</i>	0.7	0.8	0.5	1.1		
448	<i>Uncultured Ambiospora</i>	5.7	6.5	4.4	7.6		
451	<i>Uncultured Ambiospora</i>	0.4	0.1	0.2	0.3		
523	<i>Glomus clarum</i>	0.8	0.7	0.3	1.0		
524	<i>Glomus clarum</i>	7.4	5.6	6.5	6.8		
525	<i>Glomus clarum</i>	8.0	5.1	5.0	5.9		
528	<i>Diversispora aurantia</i>	15.8	9.7	10.9	6.9		
531	<i>Diversispora aurantia</i>	1.9	7.3	3.8	2.3		
535	<i>Claroideoglossum lamellosum</i>	4.4	7.2	7.4	4.8		
536	<i>Claroideoglossum lamellosum</i>	12.3	13.6	12.3	9.7		
537	<i>Claroideoglossum lamellosum</i>	2.8	5.8	5.2	3.4		
375	<i>Claroideoglossum lamellosum</i>	5.7	4.0	2.9	1.8		
377	<i>Claroideoglossum lamellosum</i>	3.1	2.9	1.8	2.6		

The AMF genotype accounting for the greatest SIMPER variability was *P. laccatum* (TRF 369), which accounted for 16.9 % of dissimilarity between time

points. Other important taxa contributing to dissimilarity (%), were ranked in the following order: *D. aurantia* (TRF 528, 11.31 %), *G. iranicum* (TRF 395, 10.90 %), *C. lamellosum* (TRF 536, 8.8 %), and Uncultured *Ambispora* (TRF 448, 8.13 %). *G. iranicum* TRF 395 was particularly variable, which increased from 0.84 % to 11.4 % relative abundance, and 13.4 % to 82.8 % persistence, between Oct-10 and Oct-11 respectively (**Figure 3.8**).

The mean relative abundance of AMF genotypes, grouped by assigned taxonomy, was highly variable over time. *C. lamellosum* and *P. laccatum* mean relative abundance peaked in summer months. Relative abundance of *C. lamellosum* rose from 7.1 % in Oct-10, to 33.5 % in Jun-11, 29.6 % in Aug-11, then fell back down to 10.8 % in Oct-11. Relative abundance of *P. laccatum* increased from 16.2 % in Oct-10, to 20.1 % in Jun-11, 27.0 % in Aug-11, and decreased to 13.7 % in Oct-11. Conversely, relative abundance of *Uncultured Ambispora* and *D. aurantia* dipped in summer months. *Uncultured Ambispora* mean relative abundance dropped from 37.2 % in Oct-10, to 7.6 % in Jun-11, 5.6 % in Aug-11, and back up to 27.1 % in Oct-11. Relative abundance of *D. aurantia* fell from 21.1 % in Oct-10, to 17.0 in Jun-11, 14.6 in Aug-11, and then increased back up to 25.8 in Oct-11. Finally, the mean relative abundance of *G. iranicum* gradually increased over time from 6.0 % to 14.6 %, while that of *G. Clarum* decreased over time from 11.8 % to 4.8 %. (**Figure 3.8**).

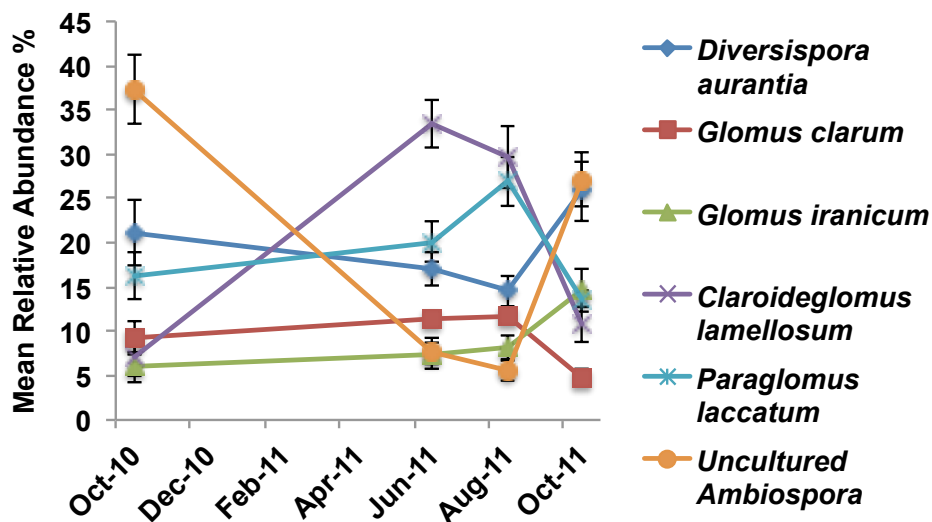


Figure 3.8 Mean relative abundance of the six AMF species, grouped into genotypes with the same taxonomic assignment, across time.

3.3.3.5 Distance decay of AMF communities across a field

There was a significant distance decay relationship, of AMF community structure, in August 2011 ($S_{BC} = 0.69D^{-0.07}$, $r^2 = 0.081$, $P = 0.002$ *). However, there was not a significant distance decay relationship in October 2010 ($S_{BC} = 0.45D^{-0.077}$, $r^2 = 0.024$, $P = 0.059$), June 2011 ($S_{BC} = 0.60D^{-0.028}$, $r^2 = 0.0201$, $P = 0.086$), or October 2011 ($S_{BC} = 0.53D^{-0.033}$, $r^2 = 0.018$, $P = 0.64$) (**Figure 3.9**).

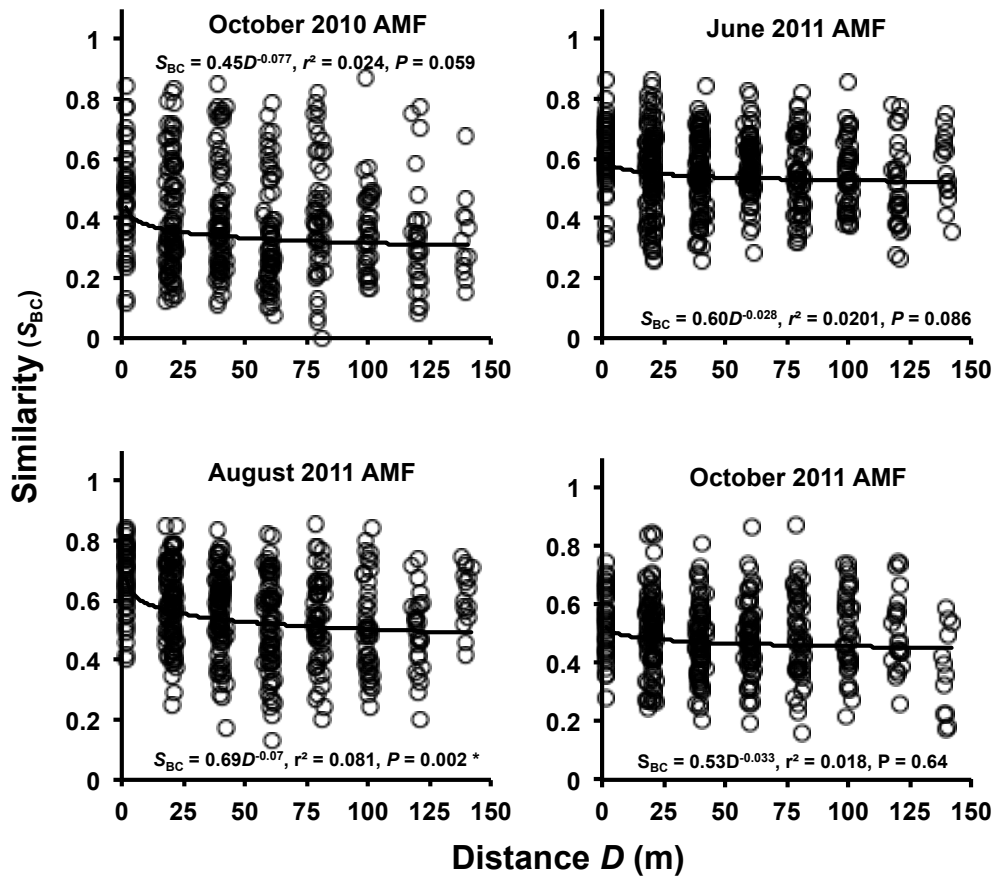


Figure 3.9 Pairwise total fungal and AMF community similarity, at increasing geographic distance, with Bray Curtis indices of similarity. In each instance, given is the distance-decay relationship power law equation, $S = cD^d$. * = $P < 0.01$. Mantel and partial Mantel summary statistics are given in **Table 3.12**.

3.3.3.6 Influence of geographic distance and environmental distance on AMF community structure

Accumulating all time points, environmental variables were driving AMF community structure ($r = 0.154$, $p = 0.004$ *), and geographic distance was not (**Table 3.11**).

Table 3.11 Summary statistics of Mantel tests across all time points, correlating community structure (Bray Curtis similarity distance matrix of TRF relative abundances) with Geo (Euclidian geographic dissimilarity matrix), and Env (Euclidian environmental dissimilarity matrix of pH, % N, % C, NO₃, NH₄, P, K, Mg, and C:N). Geo(Env) represents the partial Mantel test between geographical distance and community structure, controlling for environmental variability. Env(Geo) represents the partial Mantel test between environmental distance and community structure, controlling for Geographical variability. * = $P < 0.01$.

Factor	AMF	
	r	p
Geo	0.293	0.001*
Env	0.166	0.001*
Geo(Env)	0.007	0.407
Env(Geo)	0.154	0.004*

3.3.3.7 The influence of environmental factors on AMF community structure

Variability in AMF composition was influenced by environmental factors, rather than geographic distance, in Oct-10 and Aug-11 (**Table 3.12**). However, in June 2011 and October 2011, AMF composition was not significantly influenced by distance or environmental factors (**Table 3.12**).

In Oct-10, residual variability in %N ($r^2 = 0.318$, $P = 0.001^*$) and Mg ($r^2 = 0.302$, $P = 0.001^*$), significantly correlated with AMF composition (**Table 3.12**). However, Mg significantly correlated with %N ($r^2 = 0.73$, $P = <0.0001^*$). In Aug-11, residual variability in pH ($r^2 = 0.331$, $P = 0.003^*$) and Mg ($r^2 = 0.427$, $P = 0.001^*$), all accounted for variability when distance was controlled for. However, Mg was more influential than distance ($r^2 = 0.005$, $P = 0.460$), and distance was still significant when controlling for pH ($r^2 = 0.196$, $P = 0.006^*$) (**Table 3.12**). Mg significantly correlated with pH ($r^2 = 0.77$, $P = <0.0001^*$). Mg was significantly correlated to AMF structure in Oct-11 ($r^2 = 0.208$, $P = 0.025$), but not below the 1 % p-value threshold. Therefore, AMF community structure was mainly affected by Mg.

Table 3.12 Distance decay statistics for AMF community structure. Summary of Mantel and Partial Mantel tests, using Bray Curtis indices of similarity, whereby * $p < 0.01$ significance.

AMF		Oct-10		Jun-11		Aug-11		Oct-11	
Parameter	Control For	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Distance		0.133	0.059	0.091	0.086	0.233	0.002 *	0.095	0.064
Distance	Env	0.064	0.210	0.072	0.149	0.003	0.463	0.004	0.448
Distance	pH	0.136	0.030	0.055	0.143	0.196	0.006 *	0.063	0.152
Distance	%C	0.081	0.146	0.121	0.050	0.150	0.029	0.107	0.092
Distance	%N	-0.038	0.686	0.101	0.104	0.135	0.041	0.151	0.018
Distance	NO ₃	0.138	0.038	0.080	0.099	0.159	0.020	0.067	0.137
Distance	NH ₄	0.150	0.023	0.067	0.170	0.240	0.001 *	0.090	0.077
Distance	K	0.129	0.051	0.092	0.099	0.233	0.001 *	0.105	0.060
Distance	Mg	-0.011	0.531	0.069	0.160	0.005	0.460	-0.009	0.536
Distance	P	0.200	0.017	0.077	0.109	0.245	0.002 *	0.069	0.148
Env		0.221	0.024	0.064	0.287	0.471	0.001 *	0.202	0.012
Env	Distance	0.189	0.054	0.031	0.393	0.421	0.001 *	0.179	0.036
pH	Distance	-0.030	0.666	0.126	0.076	0.331	0.003 *	0.108	0.093
%C	Distance	0.178	0.068	-0.084	0.758	0.123	0.112	-0.052	0.755
%N	Distance	0.319	0.006 *	-0.046	0.656	0.072	0.202	-0.127	0.978
NO ₃	Distance	0.088	0.194	0.055	0.247	0.210	0.012	0.112	0.106
NH ₄	Distance	-0.099	0.938	0.034	0.306	-0.079	0.812	0.101	0.117
K	Distance	-0.086	0.805	0.009	0.433	-0.017	0.550	-0.120	0.949
Mg	Distance	0.302	0.001 *	0.035	0.376	0.427	0.001 *	0.208	0.025
P	Distance	-0.160	0.985	0.028	0.361	-0.087	0.846	0.099	0.130

3.3.3.8 Influence of environmental factors on AMF percent root length colonisation

The percent of AMF root length colonisation significantly positively correlated to P in Aug-11. There were no other significant correlations at $p < 1\%$ (Table 3.13).

Table 3.13 Correlations between environmental variables with AMF percent root length colonisation. Whereby, r = Pearson product-moment correlation coefficient, and * p -value < 0.01 .

Parameter	Oct-10		Jun-11		Aug-11		Oct-11	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
pH	-0.185	0.336	-0.237	0.192	0.356	0.049	0.133	0.493
N	0.449	0.015	0.086	0.639	-0.113	0.546	0.270	0.156
NO ₃	0.037	0.850	-0.163	0.373	0.077	0.679	0.050	0.797
NH ₄	0.448	0.015	0.167	0.361	-0.396	0.027	-0.127	0.511
C	0.314	0.097	0.048	0.794	-0.223	0.227	0.351	0.062
K	-0.008	0.965	0.197	0.281	-0.388	0.031	0.154	0.424
Mg	0.342	0.069	-0.132	0.471	-0.021	0.909	0.324	0.087
P	0.544	0.002*	0.182	0.319	-0.382	0.034	0.190	0.323

3.3.3.9 Influence of environmental factors on AMF taxa richness

AMF taxa richness was significantly negatively correlated with Mg in Aug-11. There were no other significant correlations at $p < 0.01$ (Table 3.14).

Table 3.14 Correlations between environmental variables with AMF TRF richness. Whereby, r = Pearson product-moment correlation coefficient, and * p-value < 0.01.

Parameter	Oct-10		Jun-11		Aug-11		Oct-11	
	r	P	r	P	r	P	r	P
pH	0.089	0.646	-0.264	0.144	-0.428	0.016	0.176	0.361
N	-0.427	0.021	-0.280	0.121	-0.340	0.061	0.047	0.809
NO ₃	-0.038	0.846	-0.335	0.061	-0.360	0.047	-0.216	0.260
NH ₄	0.048	0.806	-0.117	0.525	0.182	0.327	-0.146	0.451
C	-0.452	0.014	-0.231	0.203	-0.275	0.135	0.024	0.901
K	0.133	0.491	-0.203	0.265	0.114	0.540	-0.068	0.726
Mg	-0.278	0.144	-0.275	0.127	-0.475	0.007*	0.108	0.577
P	-0.235	0.221	-0.212	0.244	-0.205	0.269	-0.152	0.431

3.3.3.10 Influence of Mg and pH on individual AMF TRFs

Presented are only the taxa which were significantly correlated to Mg or pH (Table 3.15), as these had the strongest environmental influence on AMF community structure. There were strong positive correlations between Mg and pH with three *C. lamellosum* ribotypes: TRFs 535, 536 and 537, in Aug-11 and Oct-11. Other positive correlations with Mg and pH were a *G. iranicum* TRF (398) in Oct-10 and a *D. aurantia* TRF in Jun-11 (531). All other taxa were negatively correlated to Mg and pH (TRFs 440, 371, 372). *C. lamellosum* was the dominant AMF species structured by Mg and pH.

Table 3.15 AMF taxa (TRFs) which are significantly correlated ($p < 0.01$) to pH or Mg, across four time points. Whereby r represents the Pearson product-moment correlation coefficient.

Time	TRF	pH		TRF	Mg	
		r	P		r	P
Oct-10	537	0.639	0.000	398	0.512	0.005
	440	-0.521	0.004	535	0.575	0.001
Jun-11	371	-0.462	0.009	531	0.545	0.002
	536	0.555	0.001			
	531	0.536	0.002			
Aug-11	371	-0.534	0.002	372	-0.453	0.010
	535	0.568	0.001	398	-0.498	0.004
	536	0.615	0.000	528	-0.570	0.001
	537	0.551	0.001	535	0.616	0.000
				536	0.687	0.000
Oct-11				537	0.697	0.000
	535	0.496	0.007	535	0.600	0.001
	536	0.607	0.001	536	0.538	0.003
	537	0.449	0.017	537	0.680	0.000

3.3.4 Core and Satellite Taxa

3.3.4.1 Total fungal core and satellite taxa

Of the accumulated 548 total fungal taxa (137 TRFs in four time points), 24 taxa were core, which represented 4.4 % OTU richness and 47.6 % of all sequences. The remaining 524 were classified as satellite, which represented 95.6 % OTU richness and 52.4 % of all sequences (**Figure 3.10**).

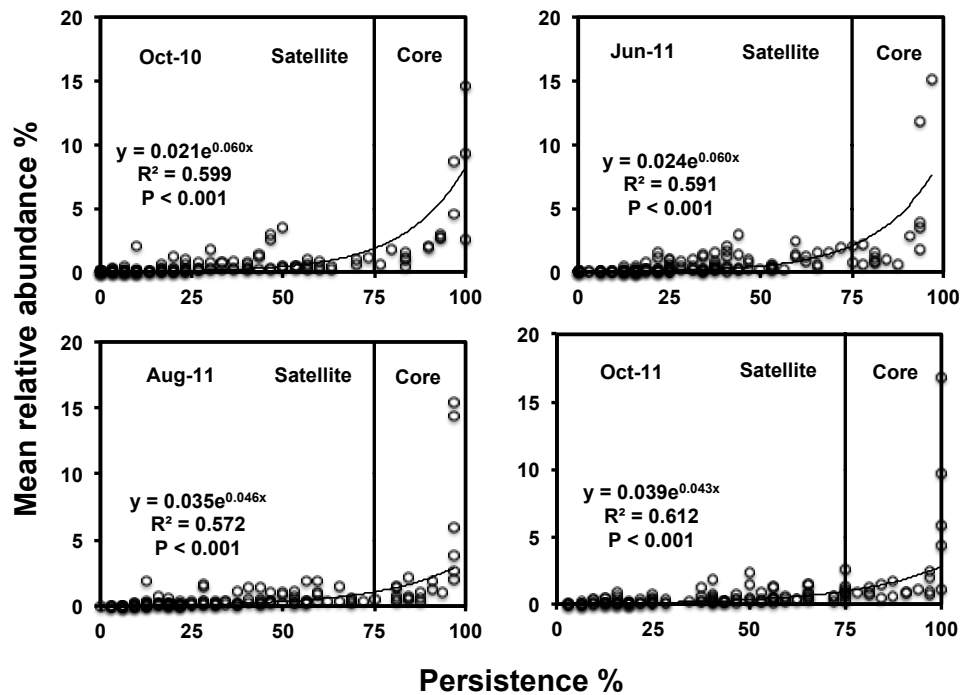


Figure 3.10 Persistence and mean relative abundance of total fungal taxa, from all samples and time points. The vertical line represents 75 % persistence. Taxa present in > 75 % samples were classified as core.

Across the four time points, ten taxa were classified as core, out of the 137 total fungal taxa (**Table 3.16**). Across time, the number and identities of core taxa changed, there were nine in Oct-10, three in Jun-11, six in Aug-11, and six in Oct-11, and only two taxa were core across all time points (TRF 140 and TRF 160). There was a large shift between Oct-10 and Jun-11, where there was a decrease from nine core taxa in Oct-11 to three in Jun-11, with only two core taxa the same between time points (TRF 140 and TRF 160), and one new core taxon appeared in Jun-11 (TRF 226). The three core taxa in Jun-11 (TRF 140, TRF 160, and TRF 226) were also core in Aug-11 and Oct-11. There were two more core taxa in Aug-11 and Oct-11 (TRF 105 and TRF 125), and a further core taxon in Oct-11 (TRF 145), all of which had been core in Oct-10, but not in Jun-11 (**Table 3.16**).

Table 3.16 Core total fungal taxa, '1' represents taxa present in >75 % samples

TRF	Oct-10	Jun-11	Aug-11	Oct-11
105	1		1	1
125	1		1	1
140	1	1	1	1
145	1			1
160	1	1	1	1
161	1			
198	1			
226		1	1	1
442	1			
494	1			
Total	9	3	5	6

3.3.4.2 Temporal scaling of core and satellite total fungal taxa

Core and satellite fungal communities significantly shifted between every time point (ANOSIM $p < 0.001$), except between Aug-11 and Oct-11 (ANOSIM $r = -0.13$, $P = 0.869$, and $r = 0.039$, $P = 0.45$ respectively **Table 3.17**). However, for core fungi, the strength (r values) of the shift was greater (**Table 3.17**), and the separation between time points was more clearly defined (**Figure 3.11**).

Table 3.17 ANOSIM summary statistics between time points, of core and satellite taxa. r^2 values are displayed in the bottom left triangle, and p values in the top right triangle. Where, * represents a significant difference at $P < 0.05$.

Core					Satellite				
$r \setminus p$	Oct-10	Jun-11	Aug-11	Oct-11	$r \setminus p$	Oct-10	Jun-11	Aug-11	Oct-11
Oct-10	x	0.001 *	0.001 *	0.001 *	Oct-10	x	0.001 *	0.001 *	0.001 *
Jun-11	0.945	x	0.001 *	0.001 *	Jun-11	0.452	x	0.001 *	0.001 *
Aug-11	0.867	0.938	x	0.869	Aug-11	0.293	0.286	x	0.450
Oct-11	0.873	0.975	-0.013	x	Oct-11	0.258	0.301	0.039	x

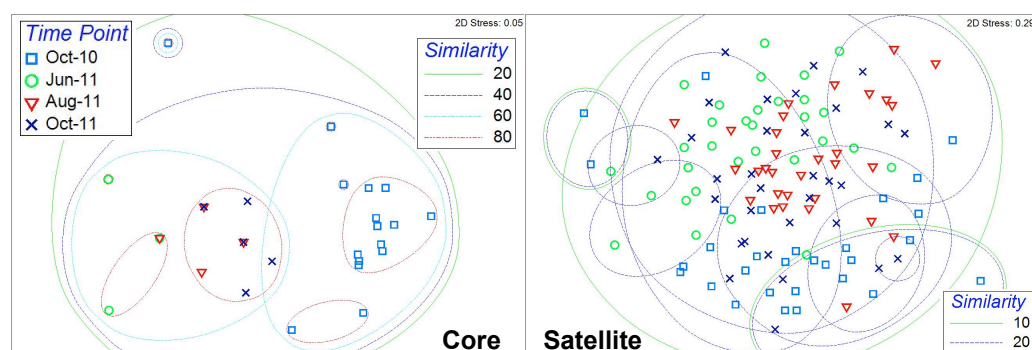


Figure 3.11 NMDS plot, using Sorensen similarity, of total fungi separated into core and satellite taxa. Oct-10 $n = 30$, Jun-11 $n = 31$, Aug-11 $n = 31$, Oct-11 $n = 32$, the number of samples for each time point looks reduced in the core NMDS plot, as many points overlap, which means the presence and absence of core taxa in those samples are exactly the same.

3.3.4.3 α -diversity of core and satellite taxa

The similarity within time points was far greater in core taxa than satellite taxa, with a mean of 90.1 % compared to 23.3 % similarity respectively (**Table 3.18**).

Table 3.18 Similarity within time points, of core and satellite taxa.

Time	Similarity %	
	Core	Satellite
Oct-10	82.6	20.6
Jun-11	94.0	26.1
Aug-11	91.2	23.7
Oct-11	92.8	22.9
Mean	90.1	23.3

3.3.4.4 Influence of environmental factors on core total fungal community structure

Overall, core total fungal taxa were not spatially structured ($r = 0.049$, $P = 0.071$), or influenced by environmental factors ($r = -0.046$, $P = 0.827$) (**Table 3.19**). Core total fungal community structure was mainly influenced by spatial distance in Jun-11 ($P < 0.001$), and pH in Oct-11 ($r = 0.267$, $P = 0.004$) (**Table 3.20**). There were no significant drivers of community structure in Oct-10 or Aug-11.

Table 3.19 Summary statistics of Mantel tests across all time points, correlating community structure (Bray Curtis similarity distance matrix of TRF relative abundances) with Geo (Euclidian geographic dissimilarity matrix), and Env (Euclidian environmental dissimilarity matrix of pH, % N, % C, NO₃, NH₄, P, K, Mg, and C:N). Geo(Env) represents the partial Mantel test between geographical distance and community structure, controlling for environmental variability. Env(Geo) represents the partial Mantel test between environmental distance and community structure, controlling for Geographical variability. * = $P < 0.01$.

Factor	Core Fungi		Satellite Fungi		Core AMF		Satellite AMF	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Geo	0.033	0.124	0.122	0.001 *	0.074	0.015	0.064	0.002 *
Env	-0.029	0.708	0.166	0.001 *	0.176	0.001 *	0.048	0.084
Geo(Env)	0.049	0.071	0.063	0.006 *	0.004	0.44	0.049	0.02
Env(Geo)	-0.046	0.827	0.13	0.001 *	0.16	0.005 *	0.023	0.262

Table 3.20 Summary of Mantel and Partial Mantel tests, for core total fungi community structure, using Bray Curtis indices of similarity, whereby * $p = < 0.01$ significance.

Parameter Control For		Oct-10		Jun-11		Aug-11		Oct-11	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Distance		-0.033	0.633	0.288	0.001 *	0.046	0.274	0.033	0.267
Distance	Env	0.009	0.435	0.333	0.001 *	0.047	0.269	-0.066	0.843
Distance	pH	-0.036	0.656	0.303	0.001 *	0.036	0.304	-0.053	0.785
Distance	%C	-0.009	0.493	0.351	0.001 *	0.053	0.247	-0.019	0.580
Distance	%N	-0.017	0.499	0.343	0.001 *	0.048	0.289	0.001	0.487
Distance	NO ₃	-0.033	0.602	0.282	0.001 *	-0.007	0.458	0.013	0.379
Distance	NH ₄	-0.038	0.624	0.288	0.001 *	0.033	0.299	0.032	0.283
Distance	K	-0.037	0.632	0.288	0.001 *	0.053	0.255	0.018	0.365
Distance	Mg	-0.024	0.550	0.323	0.001 *	0.044	0.299	-0.063	0.818
Distance	P	0.008	0.451	0.325	0.001 *	0.062	0.241	0.001	0.479
Env		-0.131	0.873	-0.041	0.634	0.010	0.424	0.198	0.068
Env	Distance	-0.127	0.843	-0.179	0.971	-0.015	0.538	0.206	0.051
pH	Distance	0.016	0.371	-0.098	0.931	0.068	0.247	0.267	0.004 *
%C	Distance	-0.073	0.668	-0.213	0.997	-0.026	0.548	0.069	0.173
%N	Distance	-0.023	0.503	-0.194	0.983	-0.019	0.525	0.039	0.295
NO ₃	Distance	0.034	0.322	0.008	0.443	0.152	0.091	0.096	0.143
NH ₄	Distance	0.027	0.345	-0.069	0.774	0.099	0.134	0.030	0.336
K	Distance	-0.156	0.945	-0.124	0.935	-0.094	0.807	0.202	0.026
Mg	Distance	-0.016	0.525	-0.154	0.943	-0.008	0.456	0.198	0.078
P	Distance	-0.059	0.666	-0.167	0.992	-0.048	0.662	0.135	0.101

3.3.4.5 Influence of environmental factors on satellite total fungal community structure

Overall, satellite total fungal communities were significantly spatially structured ($r = 0.63$, $P = 0.006$), and influenced by environmental factors ($r = 0.130$, $P = 0.003$) (**Table 3.19**). Satellite total fungal community structure was influenced by a combination of factors (**Table 3.21**). In Jun-11, community structure was influenced by geographic distance ($r = 0.225$, $P = 0.006$) and NO₃ ($r = 0.205$, $P = 0.006$). In Aug-11, community structure was mainly affected by Mg ($r = 0.321$, $P = 0.002$), but also pH ($r = 0.296$, $P = 0.001$), and NO₃ ($r = 0.318$, $P = 0.001$). In Oct-11, community structure was affected by pH ($r = 0.214$, $P = 0.001$). There were no significant correlations in Oct-10.

Table 3.21 Summary of Mantel and Partial Mantel tests, for satellite total fungi community structure, using Bray Curtis indices of similarity, where * $P < 0.01$.

Parameter Control For		Oct-10		Jun-11		Aug-11		Oct-11	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Distance		0.028	0.307	0.225	0.001 *	0.304	0.001 *	0.186	0.002 *
Distance	Env	0.019	0.395	0.172	0.006 *	0.131	0.039	0.115	0.029
Distance	pH	0.008	0.453	0.209	0.001 *	0.275	0.001 *	0.115	0.028
Distance	%C	0.026	0.327	0.153	0.015	0.258	0.002 *	0.171	0.007 *
Distance	%N	0.019	0.403	0.097	0.075	0.214	0.005 *	0.080	0.094
Distance	NO ₃	0.027	0.317	0.191	0.002 *	0.202	0.002 *	0.164	0.002 *
Distance	NH ₄	0.009	0.426	0.222	0.001 *	0.307	0.001 *	0.183	0.002 *
Distance	K	0.026	0.356	0.224	0.002 *	0.315	0.001 *	0.188	0.002 *
Distance	Mg	-0.026	0.619	0.164	0.008 *	0.129	0.038	0.113	0.042
Distance	P	0.038	0.303	0.224	0.003 *	0.352	0.001 *	0.187	0.002 *
Env		0.031	0.355	0.172	0.055	0.405	0.001 *	0.187	0.019
Env	Distance	0.023	0.400	0.091	0.186	0.308	0.003 *	0.116	0.115
pH	Distance	0.089	0.104	0.037	0.301	0.296	0.001 *	0.214	0.001 *
%C	Distance	0.001	0.498	0.090	0.165	0.011	0.428	-0.044	0.751
%N	Distance	0.011	0.441	0.169	0.055	0.044	0.273	0.105	0.045
NO ₃	Distance	-0.061	0.745	0.205	0.006 *	0.318	0.001 *	0.095	0.132
NH ₄	Distance	0.091	0.098	-0.046	0.685	-0.044	0.727	0.091	0.129
K	Distance	-0.109	0.884	-0.053	0.720	-0.146	0.966	-0.036	0.655
Mg	Distance	0.127	0.104	0.105	0.146	0.321	0.002 *	0.120	0.094
P	Distance	-0.026	0.613	-0.036	0.680	-0.190	0.991	-0.029	0.638

3.3.4.6 AMF core and satellite taxa

There were a total of twenty-one AMF taxa, and when these were accumulated over four time points, this equates to eighty-four taxa, for the purpose of assigning the number of core and satellite at each time point. In the accumulated eighty-four taxa, a total of thirty-five taxa were classified as core, which represented 40.7 % richness and 79.3 % of all sequences. There were fifty-one satellite taxa, which represented 59.3 % OTU richness and 20.7 % of all sequences, as illustrated in **Figure 3.12**.

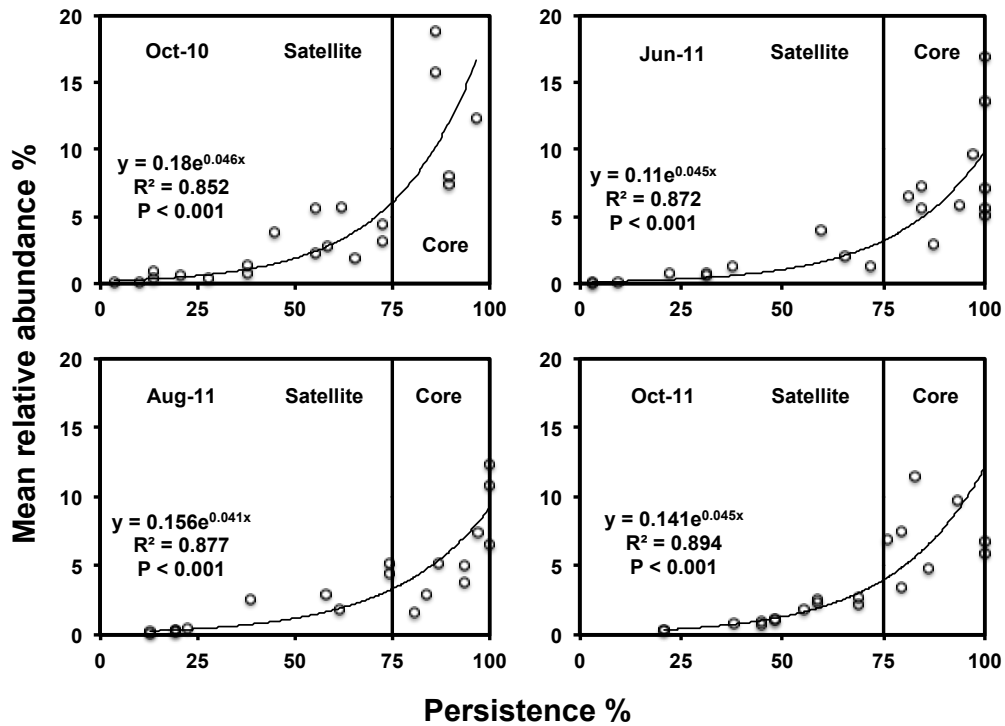


Figure 3.12 Persistence and abundance of the twenty-one AMF TRFs, from all four time points. Where the blue line is an exponential trend line, and the vertical line is the threshold for 'core' taxa at 75 % persistence.

Fourteen out of twenty-one taxa were 75 % persistent (core) in at least one time point (**Table 3.22**). Five of these were consistently core across all time points, with the remaining nine intermittently core. The greatest number of core taxa was in Jun-11 (eleven), followed by Aug-11 (ten), Oct-11 (nine) and Oct-10 (five). There were two core taxa unique to Jun-11, two core taxa unique to Aug-11, and one core taxon unique to Oct-11, and the remaining ten core taxa were in more than one time point. Between Oct-10 and Jun-11, there were six new core taxa, in Aug-11 there were two new core taxa, and in Oct-11 there was one new core taxon. On average, there were more core taxa in summer months (mean = 10.5) than autumn (mean = 7), and six taxa were only core in summer. However, six taxa were consistently core, there were no uniquely core autumn taxa common to both Octobers, some taxa became less persistence over time, and other taxa became more persistent over time. With such variability between individual taxa, the conclusion should be that changes in core taxa were temporal, not seasonal.

Table 3.22 Core total fungal taxa, '1' represents taxa present in >75 % samples

TRF	Taxa match	75 % Persistent taxa			
		Oct-10	Jun-11	Aug-11	Oct-11
369	<i>Paraglomus laccatum</i>	1	1	1	1
371	<i>Paraglomus laccatum</i>			1	
372	<i>Paraglomus laccatum</i>			1	
375	<i>Claroideglomus lamellosum</i>				
377	<i>Claroideglomus lamellosum</i>		1		
395	<i>Glomus iranicum</i>				1
398	<i>Glomus iranicum</i>		1		
401	<i>Glomus iranicum</i>				
440	<i>Uncultured Ambiospora</i>				
444	<i>Uncultured Ambiospora</i>				
445	<i>Uncultured Ambiospora</i>				
448	<i>Uncultured Ambiospora</i>		1		1
451	<i>Uncultured Ambiospora</i>				
523	<i>Glomus clarum</i>				
524	<i>Glomus clarum</i>	1	1	1	1
525	<i>Glomus clarum</i>	1	1	1	1
528	<i>Diversispora aurantia</i>	1	1	1	1
531	<i>Diversispora aurantia</i>		1	1	
535	<i>Claroideglomus lamellosum</i>		1	1	1
536	<i>Claroideglomus lamellosum</i>	1	1	1	1
537	<i>Claroideglomus lamellosum</i>		1	1	1
Total No. Core Species		5	11	10	9

3.3.4.7 Temporal scaling of core and satellite taxa

Between all time points core and satellite taxa had significant temporal shifts (ANOSIM, $p < 0.001^*$) (**Table 3.23**). The temporal shift between time points was greatest in core taxa (mean $r = 0.766$), and the smallest temporal shift was in satellite taxa (mean $r = 0.431$).

Table 3.23 ANOSIM summary between time points, of AMF taxa classified as core and satellite.

Dissimilarity Between		Core		Satellite	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Aug-11	Jun-11	0.545	0.001*	0.316	0.001*
Aug-11	Oct-10	0.882	0.001*	0.630	0.001*
Jun-11	Oct-10	0.883	0.001*	0.434	0.001*
Aug-11	Oct-11	0.785	0.001*	0.432	0.001*
Jun-11	Oct-11	0.867	0.001*	0.435	0.001*
Oct-10	Oct-11	0.634	0.001*	0.339	0.001*
Mean (\pm S.E.M)		0.766 (± 0.06)		0.431 (± 0.05)	

The clearest temporal shifts occurred in core taxa, with some time points clustered at 80 % similarity, and most samples fitted within the 60 % similarity cluster (**Figure 3.13**). Satellite taxa clustered with lower similarity (20 - 40 %) compared to core, and there was more overlap between time points. Core taxa had distinct clusters at each time point, with similarities of ~80 %, and clearly shifted over time. Satellite taxa also showed temporal shifts, but had less similarity within and between time points (40 - 60 %), compared to core taxa (**Figure 3.13**).

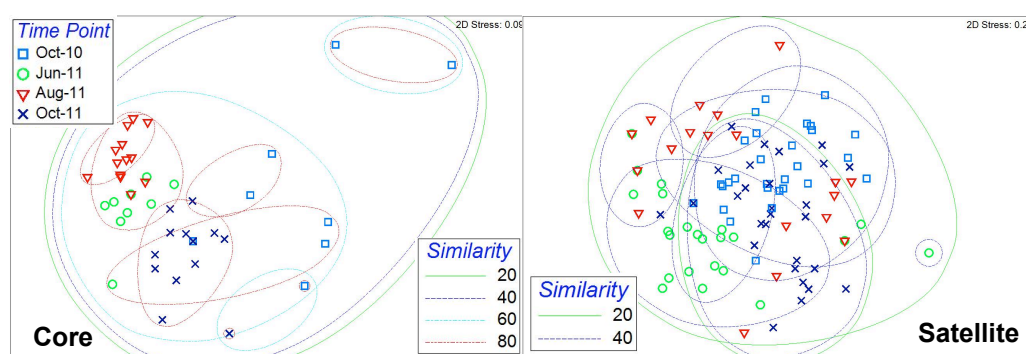


Figure 3.13 NMDS plot for core and satellite taxa (TRFs), over four time points. Clustering with Bray Curtis similarity at 20, 40, 60, and 80 %.

3.3.4.8 α -diversity of core and satellite taxa

Within time points, core taxa were more similar (88.1 %) than satellite taxa (43.8 %) (**Table 3.24**). The overall difference in similarity between core and satellite was 44.4 %.

Table 3.24 Sorensen similarity of AMF TRF relative abundance within time points, of core and satellite taxa.

Time	Similarity %	
	Core	Satellite
Oct-10	82.2	43.9
Jun-11	91.3	42.4
Aug-11	90.7	40.4
Oct-11	88.3	48.4
Mean	88.1	43.8

3.3.4.9 The influence of environmental factors on core AMF community structure

Overall, core AMF were influenced by environmental factors ($r = 0.160$, $P = 0.005$), and not geographic distance ($r = 0.004$, $P = 0.440$) (Table 3.19). Core AMF community structure was affected by a combination of factors (Table 3.25). In Aug-11 community structure was influenced by pH ($r = 0.365$, $P = 0.001$), NO_3 ($r = 0.267$, $P = 0.004$), and Mg ($r = 0.479$, $P = 0.001$). In Oct-11, community structure was mainly influenced by Mg ($r = 0.255$, $P = 0.010$). There were no significant influences found in Oct-10 or Jun-11.

Table 3.25 Distance decay statistics for core AMF community structure. Summary of Mantel and Partial Mantel tests, using Bray Curtis indices of similarity, whereby * $p < 0.01$ significance.

Parameter Control For		Oct-10		Jun-11		Aug-11		Oct-11	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Distance		0.126	0.065	0.081	0.077	0.247	0.001 *	0.082	0.102
Distance	Env	0.070	0.183	0.052	0.209	-0.011	0.536	-0.029	0.656
Distance	pH	0.140	0.025	0.038	0.254	0.210	0.005 *	0.042	0.242
Distance	%C	0.075	0.179	0.101	0.077	0.149	0.038	0.093	0.137
Distance	%N	-0.037	0.650	0.078	0.151	0.128	0.051	0.135	0.040
Distance	NO_3	0.130	0.051	0.066	0.118	0.158	0.022	0.051	0.185
Distance	NH_4	0.145	0.037	0.074	0.129	0.250	0.001 *	0.076	0.124
Distance	K	0.124	0.068	0.083	0.110	0.250	0.004 *	0.093	0.095
Distance	Mg	0.013	0.428	0.050	0.214	-0.008	0.511	-0.044	0.731
Distance	P	0.180	0.035	0.057	0.164	0.264	0.002 *	0.054	0.202
Env		0.183	0.048	0.086	0.173	0.521	0.001 *	0.235	0.017
Env	Distance	0.151	0.107	0.058	0.270	0.474	0.001 *	0.222	0.020
pH	Distance	-0.069	0.825	0.152	0.040	0.365	0.001 *	0.139	0.057
%C	Distance	0.176	0.070	-0.061	0.706	0.156	0.066	-0.046	0.710
%N	Distance	0.305	0.016	-0.019	0.538	0.102	0.131	-0.118	0.944
NO_3	Distance	0.060	0.261	0.077	0.166	0.267	0.004 *	0.124	0.094
NH_4	Distance	-0.105	0.935	-0.003	0.492	-0.036	0.615	0.133	0.065
K	Distance	-0.057	0.672	0.031	0.336	-0.046	0.637	-0.132	0.957
Mg	Distance	0.235	0.021	0.057	0.284	0.479	0.001 *	0.255	0.010 *
P	Distance	-0.134	0.954	0.060	0.219	-0.101	0.858	0.108	0.129

3.3.4.10 The influence of environmental factors on satellite AMF community structure

Overall, satellite AMF communities were influenced mostly by geographic distance ($r = 0.064$, $P = 0.002$), however, this was not the sole driver, when environmental variation was removed ($r = 0.049$, $P = 0.020$) (Table 3.19). Satellite AMF community structure was influenced by measured factors in Oct-10 and Oct-11, but not Jun-11 or Aug-11. In Oct-10, community structure was mainly influenced by % C ($r = 0.210$, $P = 0.004$), and Mg ($r = 0.197$, $P = 0.004$). Oct-11 community structure was mainly affected by geographic distance ($r = 0.193$, $P = 0.005$) (Table 3.26).

Table 3.26 Distance decay statistics for satellite AMF community structure. Summary of Mantel and Partial Mantel tests, using Bray Curtis indices of similarity, whereby * $p = < 0.01$ significance.

Parameter Control For		Oct-10		Jun-11		Aug-11		Oct-11	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Distance		0.009	0.469	0.146	0.017	0.094	0.062	0.153	0.012
Distance	Env	-0.063	0.865	0.157	0.021	0.027	0.326	0.193	0.005 *
Distance	pH	0.002	0.451	0.117	0.035	0.073	0.114	0.144	0.015
Distance	%C	-0.046	0.763	0.172	0.020	0.051	0.216	0.146	0.023
Distance	%N	-0.077	0.897	0.186	0.016	0.063	0.160	0.188	0.006 *
Distance	NO ₃	0.012	0.396	0.139	0.021	0.070	0.134	0.146	0.013
Distance	NH ₄	0.010	0.421	0.078	0.116	0.096	0.062	0.153	0.010 *
Distance	K	0.012	0.383	0.144	0.016	0.083	0.086	0.156	0.011
Distance	Mg	-0.078	0.914	0.151	0.022	0.032	0.297	0.191	0.002 *
Distance	P	0.067	0.178	0.164	0.009	0.044	0.230	0.145	0.019
Env		0.201	0.008 *	0.004	0.425	0.141	0.061	-0.042	0.691
Env	Distance	0.210	0.004 *	-0.060	0.703	0.110	0.099	-0.127	0.923
pH	Distance	0.023	0.338	0.095	0.132	0.156	0.023	0.012	0.424
%C	Distance	0.196	0.008 *	-0.092	0.837	0.065	0.183	-0.041	0.708
%N	Distance	0.175	0.013	-0.118	0.874	0.012	0.426	-0.112	0.959
NO ₃	Distance	0.043	0.262	0.020	0.359	0.056	0.210	0.009	0.460
NH ₄	Distance	-0.005	0.508	0.121	0.107	-0.029	0.627	-0.003	0.523
K	Distance	0.052	0.237	-0.018	0.526	0.147	0.026	-0.046	0.738
Mg	Distance	0.197	0.004 *	-0.046	0.631	0.102	0.130	-0.123	0.926
P	Distance	-0.103	0.935	0.083	0.850	0.087	0.122	0.014	0.431

3.4 Discussion

For both total rhizosphere fungi and AMF, core taxa were more consistent than satellite taxa, across space and time. Core and satellite taxa had different distributions, log-normal and log-series respectively, and their compositions were driven by different factors. The proportion of core AMF taxa was much greater than total rhizosphere fungi. The factors affecting the organisation of total root fungi could not be determined. In contrast, AMF composition was affected by soil properties, particularly Mg and pH. Core and satellite, AMF and total fungal communities significantly changed composition over time, and AMF colonisation was highest in summer months.

3.4.1 Proportion of core and satellite in AMF and total fungi

Both total fungi and AMF could be separated into core and satellite taxa. However, there was a ten-fold difference in the proportion of core taxa between total fungi and AMF. For total fungi the core taxa accounted for 4.4 % of taxa richness, which represented 52.4 % of all sequences, compared to the AMF core, which comprised 40.7 % taxa richness, representing 79.3 % of all sequences. Therefore, the AMF community had a different dynamic to that of total fungi, as most total fungi were uncommon and very few total fungi were

core, whereas a large proportion of AMF were common and core. The proportion of core fungal species is similar to the findings of Unterseher, *et al.* (2011), who found core species made up 12 % of phyllosphere fungal richness and 73 % of all sequences, while EcM core taxa made up 43 % of taxa richness and 96 % of all sequences, and AMF core taxa made up 40 % of taxa richness and 95 % of all sequences, although with a 50 % persistence threshold for core. Branco, *et al.* (2013), also found that mycorrhizal fungi (EcM), had a more even distribution compared to other fungi around the root, comprising 20 % of active soil fungi and representing 80 % of fungal sequence abundance, Dumbrell, *et al.* (2010) found AMF species abundance distributions (SAD's) mostly fitted log-normal distribution across 32 datasets. However, in general soil fungal datasets, log-series distribution is more normal. For example, Buee, *et al.* (2009) found that the three most abundant soil fungal OTU's represented 25 - 55 % of reads, in six different forests. These studies suggest mycorrhizal fungi have a more even distribution, which indicates that AMF have a more specialized role than other fungi in the rhizosphere.

3.4.2 Factors affecting core community structure

Core communities of total fungi and AMF were influenced by different factors. In total fungal communities, the factors which influenced core community structure could not be determined. However, in AMF communities, core taxa community structure was affected by soil properties. This is consistent with the assumption that AMF core communities are selected by the plant to improve plant nutrition, which is controlled by environmental factors and plant nutrient status. In contrast, total fungal core communities appear to have little association with soil nutrient status. A possible explanation could be the difference in location and lifestyle between general fungi and AMF. AMF live symbiotically within the root cortex, whereas most general fungi are parasites, commensals, and saprophytes inhabiting the rhizoplane. Up to 20 - 30 % of plant C is transferred directly to AMF within the roots, and the remaining root exudates are exuded at the root apex where they are assimilated by rhizosphere microbes (Dennis, *et al.*, 2010). Therefore the factors which influence growth and nutrition of symbionts inside the root, and fungi with other nutritional modes in the rhizoplane, are likely to be very different. Plants are also more in control of the symbionts they allow to inhabit the root cortex, by means of the interplay of plant hormones including strigalactones, and fungal myc-factors (Gutjahr and

Parniske, 2013; Kosuta, *et al.*, 2003). The regulation of symbionts can be attributed to nutritional status of the plant, particularly in nutrient stress (Jeffries, *et al.*, 2003; Vance, *et al.*, 2003), which could explain the influence of soil properties on AMF core communities.

3.4.3 Factors affecting satellite community structure

Total fungal and AMF satellite communities were also influenced by different factors. Both satellite communities were spatially structured, however total fungal satellite taxa were influenced by soil properties, particularly nitrate, pH, and Mg, whereas the factors structuring AMF satellite taxa could not be determined. Furthermore, as total fungal satellite taxa abundance and persistence were very low, and their communities more dissimilar than core communities, it is likely that satellite fungi were structured more by environmental factors, and not selected by plant nutritional status.

3.4.4 Stability of core and satellite

In both total fungi and AMF, core taxa had higher similarity within and between months, compared to satellite taxa. This means that core communities are more stable than satellite communities, which suggests core taxa have been selected, or they were the most competitive, in the rhizosphere. However, the higher turnover of satellite species may be due to increased rates of succession, immigration (Preston, 1960), extinction, colonisation (Hanski and Gyllenberg, 1993), or other environmental processes, including plant physiological changes.

3.4.5 AMF community drivers

AMF communities were spatially structured due to environmental variables, particularly Mg and pH. The importance of Mg, rather than P, was unexpected, as the majority of previous studies investigating the role of AMF in nutrient cycling focus on the influence of P on AMF communities (Treseder, 2004). P was significantly positively correlated with AMF root length colonisation, but only in Oct-11. Previous studies have shown that artificially increasing soil P does alter AMF community composition at certain levels (Cavagnaro, *et al.*, 2005; Gosling, *et al.*, 2013; King-Salter, *et al.*, 2007). However, in a similar environmental study of AMF communities, of different land use types across

Ireland, Hazard, *et al.* (2013) also found that P did not significantly influence AMF composition. Mg is rarely measured in biogeography studies, therefore the role of Mg limitation in determining AMF community structure on a larger scale, needs further investigation. Mg was also significantly negatively correlated with TRF richness in August-11, so that soil with high Mg concentrations were less diverse. The influence of Mg on AMF community structure in this study indicates that AMF have an important function within the roots of *M. giganteus*, potentially enhancing plant nutrition of Mg, which was a distinguishing feature compared to other root inhabiting fungi found in this study.

3.4.6 Total fungal community drivers

Total fungal community structure was not influenced by any factor measured in this study, however, the structure may have been influenced by the underlying processes determining microbial biogeographical patterns, including the interplay of factors proposed by Hanson, *et al.* (2012); selection, drift, dispersal, and mutation. Other factors which may have influenced total fungal community structure, include the spatial variability of a number of physiological drivers which influence the rhizosphere, including yield (Price, *et al.*, 2004), shoot growth (Kobayashi and Yokoi, 2003), photosynthesis (Tang, *et al.*, 1989), or nutrients in roots (Neukirchen, *et al.*, 1999). Seasonal changes have also been found in soil characteristics in *M. giganteus* cropping systems, including the abundance of fatty acids in the rhizosphere (Weisenberg, *et al.*, 2012), microbial biomass, and N, P, and K concentrations (Beale and Long, 1997; Himken, *et al.*, 1997).

3.4.7 Core and Satellite identification method

When the 75 % persistence threshold was used to identify core communities, both core and satellite taxa clearly shifted over time. Across the time points, some strains stayed assigned as core taxa, whereas other strains fluctuated between core and satellite. However, there was a clear relationship between persistence and abundance, as found in many other population studies, including fungal (Neubert, *et al.*, 2006) and bee diversity (Hanski, 1982b). The major issue with a 75 % persistence threshold is there are some taxa classified as core with low abundance. However, if these taxa are in over 75 % of root samples, even at low abundance, they are likely to be important.

3.4.8 Temporal shifts

AMF and fungal communities changed over time, supporting the theory that species distributions are continually turning over (Magurran, 2007; Preston, 1960). Morphologically, AMF colonisation was highest at the beginning of the growing season, decreasing over the year. Welsh, *et al.* (2010) also found AMF colonisation to be highest during vegetative growth, which declined during the growing season, in *Spartina patens*. Tian, *et al.* (2011) and Busby, *et al.* (2011) also found colonisation was lower in colder months. When genotypes were grouped into taxonomic identities, there appeared to be seasonal shifts in some species, including *Claroideoglossum lamellosum*, which peaked in summer months, and uncultured *Ambispora* which troughed in summer months (**Figure 3.8**). However, the dynamics of individual taxa was extremely variable over time (**Table 3.10**), with some remaining stable, and others rising or falling over time. These community dynamics appear to be more of a shift over time, than distinct seasonal shifts, which support the conclusions of a three year study by Husband, *et al.* (2002).

3.4.9 Transient core taxa

AMF core and satellite taxa were influenced by different factors at different time points, which may be an effect of transient AMF core taxa i.e. the taxa which were core in some, but not all, time points. In particular, six taxa changed classification from satellite to core between Oct-10 and Jun-11 respectively. This coincided with a shift from environment driving satellite taxa only in Oct-10, to environment driving core only in the next year (Aug-11 and Oct-11). Five of these transient core taxa were significantly correlated with Mg in partial Mantel tests, *Claroideoglossum lamellosum* (TRFs: 377, 535, 537), *Glomus iranicum* (TRF 398), and *Diversispora aurantia* (TRF 531). Transient core taxa were correlated with Mg, and Mg was the most important driver for AMF communities, which therefore indicates that certain environmental variables affect the persistence of AMF taxa, particularly Mg in this case.

3.4.10 Magnesium and pH

Magnesium was consistently an important factor in determining AMF community composition, but was also consistently correlated with pH, making it difficult to

determine which factor had more influence. The importance of pH maybe underestimated, as the variation in pH was relatively low (mean = 6.13 ± 0.3 SEM) compared to Mg, which was extremely variable across the field (mean = $200 \text{ mg/kg} \pm 90$ SEM). Additionally, An, *et al.* (2008) found in a spatial study of AMF in association with wild *Miscanthus sinensis*, that AMF phylotype abundance was not correlated to geographic distance, but rather to environmental factors, mainly pH. However, in our study Mg was the only nutrient to be more influential than geographical distance at all time points, which leads to the conclusion that Mg structured the AMF community, not geographic distance. Therefore, AMF may be important to enhance plant Mg nutrition in exchange for photosynthetic sugars, as previously found in maize (Clark and Zeto, 2000), and *Sesbania grandiflora* (Giri and Mukerji, 2004). The variants of *Claroideoglomus lamellosum* (TRFs 377, 535, and 537) consistently had the strongest positive correlation with Mg and pH, and were mainly core, which implies their importance to plant nutrition. Magnesium is important to plant health as it forms the central ion in chlorophyll molecule, and therefore photosynthesis and sugar synthesis. If AMF enhance Mg incorporation into *Miscanthus*, it could increase plant growth and the sugars available for AMF.

Chapter 4. The importance of arbuscular mycorrhizal fungi to *Miscanthus giganteus* growth and nutrition

4.1 Introduction

4.1.1 Rhizosphere microbes

Life in the rhizosphere is intimately linked to nutrient cycling and impacts on plant productivity. Rhizosphere microbes can be directly beneficial to plant growth, particularly symbionts such as nitrogen fixing bacteria and mycorrhizal fungi. A meta-analysis of 384 studies in legumes, showed that yield was improved 59 % by inoculation with N-fixing rhizobia, 45 % by inoculation with arbuscular mycorrhizal fungi (AMF), and 44 % when AMF and rhizobia were combined (Kaschuk, *et al.*, 2010). A number of elements are essential for plant growth, including macronutrients (P, N, S, Mg and Ca) and micronutrients (Cu, Cl, Fe, B, Mo, Mn, Ni and Zn) (Marschner, 2012). AMF form symbiotic associations with ~80% plant species (Smith and Read, 2008), and it is thought their main role is to enhance plant uptake of a range of nutrients, particularly P, and enhance water uptake and pathogen resistance (Morgan, *et al.*, 2005). However, other microbial processes can have negative impacts on plant growth. Nutrient assimilation and denitrification by microbes can reduce availability of growth limiting nutrients to plants (Jackson, *et al.*, 1989; Vitousek and Howarth, 1991). The rhizosphere is also home to many pathogens, which can specifically or opportunistically parasitise plants (Whipps, 2001).

4.1.2 *Miscanthus x giganteus*

Miscanthus x giganteus is a C4 grass, native to Asia and Africa. It is grown globally as a biomass crop to create energy from combustion, and covers over 15,000 ha in England (NNFCC, 2008). *M. giganteus* is a sterile hybrid, propagated from rhizomes, which are storage organs that sequester assimilates for rapid shoot growth in the next growing season (Suzuki and Stuefer, 1999). It is very water efficient and requires no nitrogen fertiliser (Finch and Riche, 2010), which may be due, in part, to the practice of leaving fallen leaf litter in the field.

M. giganteus is an untitled, perennial crop, and after 3 - 5 years establishment, shoots are harvested annually, for up to 20 years. Stability of land use, long highly productive growing seasons (Haughton, *et al.*, 2009), and carbon inputs into the soil from *M. giganteus*, are likely to promote stable microbial communities in the rhizosphere. AMF have been found in wild *Miscanthus sinensis* species in Japan (Murakoshi, *et al.*, 1998), however there is no understanding of the importance of AMF to *M. giganteus* growth and nutrition, in the wild or when it is grown in bioenergy cropping systems.

4.1.3 AMF-free control

Simple systems suggest AM fungi can benefit host growth (Hoeksema, *et al.*, 2010), however understanding of their role under real environmental conditions is limited because it is very difficult to set up AMF free controls. A number of methods have been used to study the importance of AMF for plant growth and nutrition. However there are difficulties with each method, due to the obligate lifestyle of AMF with the host, and the complex microbial diversity in soil. To create an AMF-free control, researchers have mainly either sterilised soil completely then added inoculum back in for the AMF treatment, or have applied fungicide to reduce abundance of AMF populations.

4.1.4 Soil sterilization and AMF inoculation

It is important that soil sterilisation does not change its characteristics so that the system is maintained in a state as close to real conditions as possible. However, this is problematic, and it is particularly challenging to maintain the physical and chemical characteristics of the soil following sterilisation. Additionally, completely sterilising large volumes of soil is difficult, and residual microbial cells and spores may germinate later under suitable conditions (Trevors, 1996). There are three commonly used soil sterilization treatments, which have been used to create an AMF-free control: γ -irradiation, autoclaving, and chemical sterilization. Sterilising soil by autoclaving or γ -irradiation can result in alterations of soil characteristics, such as decreased soil aggregation and increased dissolved organic matter and nutrient availability, which alters soil biota and plant responses (Berns, *et al.*, 2008; De Deyn, *et al.*, 2004). Since AMF inocula are generally from cultures which contain other bacteria and fungi, control soils need to be inoculated with this background biota to provide a robust non-AMF

control. The background biota can be created by washing AMF spores held on a filter, as AMF spores they are larger than most bacteria and fungal hyphae (Koide and Li, 1989).

4.1.5 Fungicides

Another way to create an AMF free control is to use fungicide. Fungicides are useful to control fungi, but most fungicides will target multiple fungal groups, and none have been found to target AMF specifically (Kahiluoto and Vestberg, 2000). Therefore, using a fungicide to suppress AMF populations will ultimately alter the whole fungal community structure, and needs to be considered when interpreting results. Fungicides can have indirect positive or negative effects on bacteria and plants (Scheepmaker and van de Kastele, 2011). Therefore the ideal fungicide would have a strong suppressive impact on AMF, but minimal toxicity and potential to stimulate growth of plants and other microbes, including saprophytic fungi, in the system. A literature review was carried out to find the most effective fungicide for this purpose.

4.1.6 Literature review of fungicide impacts on AMF colonisation

A literature review was conducted, to collate data on the impact of different pesticides on root colonization by AMF. An 'AMF reduction experiment' was defined as an experiment where fungicide was used to reduce the populations of AMF in a plant growth system, and either the AMF colonisation, or plant growth response, or both, were measured and compared to a control. A total of 171 AMF reduction experiments were collated from literature investigating the impact of pesticides on AMF colonization. This included data on fifty-two different pesticides and sixty-eight different host plant species, across thirty-four studies (**Appendix Table 4.1**). Some studies measured the impact of many fungicides on one host species (Hernandez-Dorrego and Pares, 2010), whereas other studies analysed the effect of one fungicide on many host species (Gange, *et al.*, 1990). Some studies measured the impact of fungicides on AMF colonisation or plant growth (Hernandez-Dorrego and Pares, 2010), while others investigated the use of AMF for applications such as decontaminating soils (Kangwankraiphaisan, *et al.*, 2013). All of the pesticides analysed were fungicides, with exception of three insecticides (Ipsilantis, *et al.*, 2012) and a fertilizer (Parvathi, *et al.*, 1985), and were tested at various doses, from half to

one hundred times the recommended agricultural dose, as listed in **Appendix Table 4.1**.

4.1.7 Host response

Application of fungicide elicited a range of host responses, from stunting to stimulation, but the main effect was to reduce plant growth. A number of other effects of the fungicides were found, including altered leaf macronutrient (P and N) (Karanika, *et al.*, 2008; Pedersen and Sylvia, 1997) and micronutrient (Zn) concentrations (Kangwankraiphaisan, *et al.*, 2013), plant species composition (Dostalek, *et al.*, 2013), flowering and bee visits (Cahill, *et al.*, 2008). However, in most studies, it was difficult to interpret whether the host response was the result of reduced AMF colonisation, or direct effects of the pesticide, as suitable controls were not in place, except for Jin, *et al.* (2013), where fungicide was applied to plants with and without AMF inoculation. In addition, many papers did not measure host response, or were only looking for specific factors, such as plant density or P levels. Therefore, due to the variation in aims between different studies, the first criterion for identifying a suitable fungicide, was to find the most effective at suppressing AMF.

4.1.8 Colonisation response

The effect of fungicides on root length colonisation (% RLC) ranged from completely eliminating AMF, for example Benomyl in many host species (Camargo-Ricalde, *et al.*, 2010; Daleo, *et al.*, 2008; O'Connor, *et al.*, 2002), and Fenarimole, Mancozeb and Iprodione in one study with *Allium porrum* (Hernandez-Dorrego and Pares, 2010), to increasing AMF colonisation, for instance in the case of Captan (Jin, *et al.*, 2013; Sreenivasa and Bagyaraj, 1989) (**Appendix Table 4.1**). Most fungicides reduced AMF colonisation significantly, but did not result in total elimination, while in many cases community structure of AMF was altered (Ipsilantis, *et al.*, 2012; Jin, *et al.*, 2013). The most effective fungicide at reducing AMF colonisation over a range of hosts was Benomyl.

4.1.9 Potential AMF suppressors

4.1.9.1 Azoxystrobin

Azoxystrobin is a widely used, strobilurin fungicide (which accounts for 10 % of global fungicide sales), with preventative and curative properties. Azoxystrobin binds to mitochondria, stopping ATP synthesis, and is particularly effective at inhibiting spore germination and mycelial growth. However once the fungus is growing inside plant tissues Azoxystrobin has little effect (Balba, 2007). Therefore Azoxystrobin may be effective at reducing AMF spore germination and growth, but only before host colonisation. Azoxystrobin is a broad-spectrum fungicide, with action against many pathogens and soil fungus growth, but has little effect on bacterial community structure (Adetutu, *et al.*, 2008), which means it could selectively reduce fungi for an AMF control, and leave other microbes relatively undisturbed. Azoxystrobin significantly reduced AMF colonisation at its recommended field dose in *Allium porrum* (Hernandez-Dorrego and Pares, 2010) (**Table 4.1. Appendix**). However, Azoxystrobin did not eliminate mycorrhizal fungi (Hernandez-Dorrego and Pares, 2010), and low doses have been found to increase colonisation (Ipsilantis, *et al.*, 2012) (**Table 4.1. Appendix**). There is evidence that this compound also directly stimulates plant growth, and delays senescence, by enhancing antioxidant enzymes (Zhang, *et al.*, 2010). Therefore Azoxystrobin may not be suitable to reduce AMF, as it only slightly alters AMF community structure, and would have the confounding effect of stimulating plant growth.

4.1.9.2 Mancozeb

Mancozeb is one of the most commercially important organic pesticides, and has been developed for use on over 70 crops (Gullino, *et al.*, 2010). It has multi-site action and controls a broad range of pathogens, by interfering with six different biochemical processes in fungal mitochondria and cell cytoplasm, and it also inhibits spore germination (Sijpesteijn, 1984), which suggests it could be used to create an AMF control by inhibiting AMF spore germination. Mancozeb breaks down in water and has an excellent record of crop safety (Crane, *et al.*, 1997; Gullino, *et al.*, 2010), which indicates minimal phytotoxicity and value for generating AMF control treatments. Mancozeb was able to reduce AMF root length colonised (RLC) from 85 % to 0 % at the recommended dose for *Allium porrum* (Hernandez-Dorrego and Pares, 2010), and at 100 mg/kg (10x

recommended dose) for *Arcachis hypogaea* (Parvathi, *et al.*, 1985) (**Table 4.1 Appendix**). However, it only lowered RLC to 69 % in *Chloris gayana* (Sreenivasa and Bagyaraj, 1989), and to 24 - 32 % RLC in *Arachis hypogaea* (Parvathi, *et al.*, 1985) (Table 4.1 Appendix). It has also been used to kill hyperparasites on mycorrhizal spores, as it did not inhibit germination of AMF (Daniels and Menge, 1980). As a broad-spectrum fungicide, it would kill most fungi on the roots, but may not be very effective on AMF.

4.1.9.3 Iprodione

Iprodione is a granular fungicide, which can be washed into the soil. It was the focus of an investigation into alternative fungicides to Benomyl, for creating an AMF-free control (Gange, *et al.*, 1990). Iprodione was successful at eliminating AMF in *Allium porrum* at the recommended dose (Hernandez-Dorrego and Pares, 2010), and it lowered AMF colonisation of *Plantago lanceolata* from 45 % to 8 % (Gange and West, 1994) (**Table 4.1 Appendix**), which made it a candidate for creating AMF controls. However it only reduced colonisation in eight plant species and had no effect on four hosts (Gange, *et al.*, 1990) (**Table 4.1 Appendix**), therefore it may not be very effective at repressing AMF.

4.1.9.4 Gypsum

Gypsum has been shown to eliminate AMF colonisation in *Arachis hypogaea* (Parvathi, *et al.*, 1985) (**Table 4.1 Appendix**), which means it could be used for preparing AMF free treatments. However, Gypsum is made from calcium sulphate, and could act as a plant growth stimulator, confounding results. Also, the mechanism for AMF suppression by Gypsum is not well studied.

Although a number of fungicides have been found to reduce AMF colonization of roots, benomyl was the most widely used in the literature (**Table 4.1 Appendix**), and appears to be the most effective means for controlling AMF in order to measure the importance of AMF in numerous systems, with minimal effects on the plant and other microbes (Kahiluoto, *et al.*, 2000).

4.1.9.5 Benomyl

Benomyl (methyl-1-(butyl-carbamoyl)-2-benzimidazole) is a systematic, broad spectrum, organic fungicide. It was previously widely used in agriculture to combat a range of fungi. It was voluntarily phased out after a suspected contamination in 1991 lead to lengthy legal challenges about possible damages to ornamental plants, but is still used in some parts of the world (EPA, 2001). Benomyl eliminated AMF colonisation in ten studies, massively reduced RLC in thirty-three studies, and had no effect in five studies. Colonisation was mainly prevented when benomyl was applied to plants with no prior AMF colonisation, however after weeks or months, AMF colonisation often became established (**Table 4.1 Appendix**). Kahiluoto and Vestberg (2000) investigated the optimum concentration and time to apply benomyl to suppress AMF colonisation in *Linum usitatissimum*, and concluded that at least 20 mg of benomyl per 1 kg of soil (mg/kg) was needed, one week before sowing. The best strategy in the literature to ensure AMF suppression, was to apply benomyl to the soil before AMF colonisation, at a suitably high dose.

4.1.9.6 Benomyl effects on other microbes

Benomyl is a broad spectrum fungicide, used as a foliar spray. It has limited effect on non-target microbes, including fungal and bacterial communities (Callaway, *et al.*, 2004; Welc, *et al.*, 2010). Carbendazim, a break down product of benomyl, is active against Ascomycota, but less effective against Zygomycota, as there is lower binding affinity (Summerbell, 1988), which could cause a selection for Zygomycota. However, application of benomyl (50 mg/kg) to the weed *Centaurea maculosa*, reduced AMF biomass, but did not significantly affect other fungi or bacteria Callaway, *et al.* (2004). Cahill, *et al.* (2008) also found benomyl (16.7 mg/kg) had no effect on non-AMF fungi.

4.1.9.7 Benomyl Phytotoxicity

There is evidence that benomyl can be phytotoxic at certain levels. Benomyl breaks down quickly, in heat, moisture, UV light, and some solvents, into a range of compounds (Calmon and Sayag, 1976). Some breakdown products boost plant growth, including a cytokine-type activity in soy (Skene, 1972). A major breakdown product of benomyl is carbendazim (methyl 2-

benzimidazolecarbamate, MBC), which selectively binds to and inhibits fungal microtubule assembly, and only slightly affects mammalian and plant tubulin synthesis, as potency is much lower in non-fungal targets (Davidse, 1986). Garcia, *et al.* (2002) found that at a lower than recommended dose, carbendazim, improved growth and nutrition of *Nicotiana tabacum*, but at high level caused decreased weight, chlorotic burns and necrotic patches. Another decomposition product of benomyl is a herbicidal compound, N,N⁰-dibutylurea (DBU), which makes up around 1 - 5 % of total breakdown products depending on storage temperature, and is thought to be the cause of benomyl's observed phytotoxicity (Ho, *et al.*, 2007). Shilling, *et al.* (1994) found that DBU inhibited growth of cucumber at 1.25 mg/kg, but there was no effect on corn roots, even at 9.6 mg/kg DBU, which equates to 192 - 960 mg/kg benomyl if there is conversion to DBU of 1 - 5 %. Some argue that the phytotoxicity of benomyl is relatively weak and rare (Kahiluoto, *et al.*, 2000). Daleo, *et al.* (2008) found that benomyl reduced root AMF colonization at 100 mg/kg, which was associated with decreased growth of *Spartina densiflora* but increased growth of closely related, but non-mycorrhizal, *S. alterniflora*. This suggests the effect of benomyl application was to suppress AMF, rather than to damage plant growth. *Spartina* sp. and *Miscanthus* are both in the Poaceae family, therefore phytotoxic potency may be similar. Paul, *et al.* (2001) tested increasing levels of benomyl on *Arabidopsis thaliana* seeds grown on agar with fungal spores, to find a concentration which was fungicidal but not phytotoxic, and found 5 mg/kg to be optimum. However, the study was carried out on agar, which has different binding properties to soil, with a non-AMF plant, tackling non-AMF fungal pathogens, therefore the study lacks relevance to the current context. However, this study did demonstrate that an optimum concentration of benomyl can be found - a concentration which can reduce fungal populations, while having minimal phytotoxicity.

4.1.10 Aims

Numerous studies have used benomyl to reduce AMF colonization in order to quantify the contribution of AMF to plant growth, (**Table 4.1 Appendix**), but most have not tested the phytotoxicity of benomyl, which puts into question the validity of these trials. Therefore the present study aimed to first investigate the phytotoxicity of benomyl to *M. giganteus*, in **Experiment 1. The experimental determination of the phytotoxicity of benomyl to *M. giganteus***. Then, with a

non phytotoxic concentration of benomyl which is able to reduce AMF, to investigate the role of AMF in *M. giganteus* growth and nutrition in **Experiment 2. The reduction of AMF using benomyl fungicide.** Additionally, the contribution of AMF to *M. giganteus* growth and nutrition was investigated by inoculating AMF spores cultures into a sterile medium, in **Experiment 3. AMF inoculation experiment.**

4.2 Methods

4.2.1 Identifying concentrations of Benomyl to use

Some studies (**Appendix Table 4.1**) used excessively high concentrations of fungicides, with no suitable control, to determine whether the impact of benomyl on plant growth was due to reduced AMF or phytotoxic effects (Gill, *et al.*, 2013). Studies that used the recommended dose of benomyl were less likely to cause collateral damage to the plant, but also less likely to eliminate AMF colonisation. The mean concentration (\pm SEM) used in the fifty-eight benomyl studies was 68.4 (\pm 16.2) mg/kg, and the median was 42 mg/kg. Across the fifty eight studies, benomyl reduced AMF root length colonisation by a mean (\pm SEM) of 29.3 % (\pm 7.2), 63.6 % (\pm 0), 73.1 % (\pm 6.7), and 61.3 % (\pm SEM 14.1) at concentrations under 20 mg/kg, 20 – 39 mg/kg, 40 – 60 mg/kg, and 61 - 667 mg/kg, respectively. This indicates that the optimum suppressant activity was between 40 – 60 mg/kg in these studies. The most commonly used, and the optimum suppressant, concentration of benomyl in the literature was between 40 – 70 mg/kg. However, the potency of benomyl is affected by the host plant, fungal endophytes, and soil type (Spokes, *et al.*, 1981), therefore the optimum concentration for *M. giganteus* needs to be determined experimentally.

4.2.2 Experiment 1. The experimental determination of the phytotoxicity of benomyl to *M. giganteus*

To find the maximum concentration of benomyl which causes no collateral damage to *M. giganteus*, an experiment was carried out to measure the phytotoxicity of benomyl. *M. giganteus* was grown for 10 weeks, with a soil drench of benomyl at concentrations between 0 – 120 mg/kg. Experimental work

was carried out in New Energy Farm's commercial research and development glasshouse, in Leamington, Ontario (Canada), in September 2013.

4.2.2.1 Treatments and measurements

Benlate (50 % benomyl, DuPont) was dissolved in the amount of water required to remoisten Agro Mix Germinating Mix (Fafard, Quebec) to 50 % moisture. The Agro Mix contained fibromoss, vermiculite, perlite, biosol compost (made with peat, seaweed and shrimp), coco husk fiber, and limestone, with the following properties: pH 6 (± 0.5), nitrate-N 25.9 (± 6.5) mg/m³, Ca 64.6 (± 32.3) mg/m³, Mg 38.8 (± 19.4) mg/m³, P 38.8 (± 19.4) mg/m³, K > 194 mg/m³. Eight concentrations of benomyl were applied and thoroughly mixed into the Agro Mix, at: 0, 3.75, 7.5, 15, 30, 60, 90, and 120 mg/kg. To each 4 x 4 x 6 cm pot with drainage holes, 40 g of compost was added. After benomyl was added, the compost was left for three days to allow the benomyl to act, and to avoid any phytotoxic effects of applying fungicide directly to the rhizome. There were 32 replicates per treatment, which were organised into blocks of four replicates, in an augmented Latin square (**Figure 4.1**). Newly emerged rhizomes were cut from two year old micro-propagated rhizomes of *M. giganteus*. Roots were removed, and then rhizomes were washed and weighed. All rhizomes were trimmed to a standard weight of 0.5 g (± 0.1 g). One mini rhizome, with one visible bud, was planted into each pot. Plants were watered daily, and fertilised weekly with ¼ strength Rorison's nutrient solution. After 10 weeks growth, shoot and root, fresh weights and dry weights were measured.

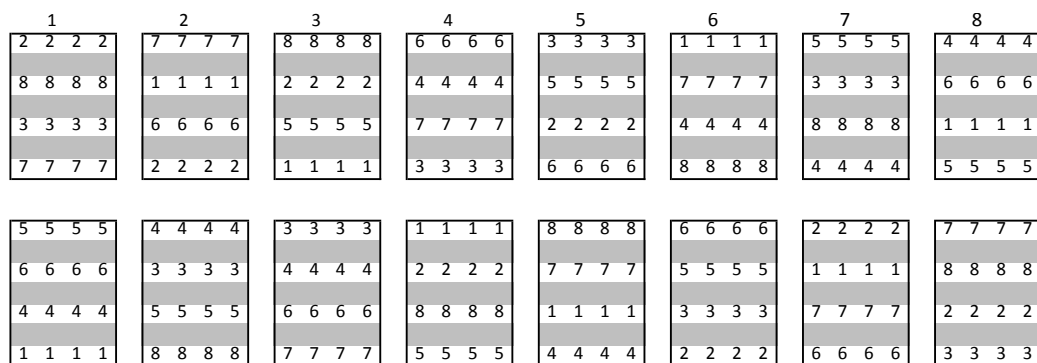


Figure 4.1 Organisation of pots into an augmented Latin square, comprising of eight blocks with four replicates. There were eight concentrations of benomyl between 0 - 120 mg/kg. Each number represents a treatment 1-8 respectively. Grey squares are empty cells in the holding tray, which were left empty to avoid cross contamination.

4.2.2.2 Statistical analysis

All statistical analysis was carried out using Genstat or Excel. Fresh and dry biomass of shoot, roots, and rhizome, were analysed for variance between treatments using a multivariate ANOVA with block structure, using raw and log-transformed data. The relationship between viable growth and benomyl concentration was analysed using linear regression.

4.2.3 Experiment 2. The reduction of AMF using benomyl fungicide

To determine the importance of AMF for host growth and nutrition, benomyl was applied to *M. giganteus* growing in pots of field soil, to suppress field populations of AMF. This experiment was established at the Warwick Crop Centre, University of Warwick, Wellesbourne, UK, in April 2011.

4.2.3.1 Soil treatment

Soil was collected from a commercial *M. giganteus* field established in 2007, near Brattleby, Lincolnshire. Site coordinates are SK 94225, BNG 81108. The soil was a sandy loam with 53 % sand, 15 % clay, and 32 % silt, pH 6.13 (± 0.3), % C 1.84 (± 1.9), % N 0.13 (± 0.09), NO₃ mg/kg 2.02 (± 1.7), NH₄ mg/kg 3.10 (± 1.6), K mg/kg 109.4 (± 35), Mg mg/kg (± 90), P mg/kg 31.4 (± 8.7). Soil was left to dry out partially for three days, then mechanically sieved to 2 mm, and homogenized thoroughly in a clean 94 L plastic bin. Soil was then divided into ten autoclave bags, 7 Kg in each. Soil moisture was measured with a soil water probe (ML2X ThetaProbe, Delta Instruments) and the amount of water required to remoisten to field moisture (25 %) was measured out. Benomyl, at 60 mg/kg, was added to the water, and 10 ml acetone was used to help dissolve the benomyl. The rehydration solution was thoroughly mixed into the soil, to ensure an even distribution of benomyl. Treated soil was left for three days to allow benomyl to act. After 3 days, the soil water probe was used to determine the water content of the soil, which was then rehydrated to 25 % field moisture content. Each pot was held by a saucer and contained draining holes. Water was added to the saucer rather than the soil, to allow uptake by roots and to prevent soil compaction.

4.2.3.2 Soil inoculation

As the soil was partially dried and sieved, viability of spores in the soil may have been reduced relative to field conditions. Therefore fresh *Miscanthus* roots and soil from unsieved Brattleby soil were added to each pot to increase the chance of colonization. Root inoculations were added to both treatments. Roots and adhering soil were taken from the top 0 – 30 cm of soil from a commercial *Miscanthus* field, near Brattleby in Lincolnshire, and cut into 1cm pieces. Twenty-five grams of soil and roots were used to inoculate each pot of soil.

4.2.3.3 Rhizomes

Commercial rhizomes were donated by New Energy Farms (Taunton, UK), and stored at 5 °C until processed. Fifty rhizomes were delivered with soil and roots attached. The roots were cut off to avoid contamination by any AMF present in the roots, and the soil washed off, taking care not to damage the rhizome. Twenty rhizomes were selected with the most similar length, shape, and number of healthy pink buds. Rhizomes ranged in weight between 20.0 - 32.2 g. To reduce variability, biomass was trimmed using a Stanley knife to ensure that all rhizomes weighed between 20 – 25 g, and possessed four healthy looking buds. Rhizomes were placed in 5 cm deep trenches with buds facing up, and were then covered in soil.

4.2.3.4 Experimental design

Five replicates of both control and benomyl treatments were prepared. Pots were organised into an augmented Latin square block design (**Figure 4.2**), and left to grow for 6 months in a temperature controlled glasshouse.

4.2.3.5 Measurements

During the experiment, every two weeks, leaf number, stem height, and plant height were measured. After ten weeks growth, photosynthesis was analysed using a CIRAS-1 IRGA (PP Systems), to measure CO₂ flux through leaves at ambient CO₂ concentration (360 ppm). IRGA measurements were taken on the youngest fully expanded leaf, on five consecutive days, between 9 - 11 am to

reduce variability between days. At 11 weeks, a subsample of roots was taken from each pot to estimate root length colonisation by AMF.





















				
				
				
				

Figure 4.2 Augmented Latin square design of pots in glasshouse, where A = controls, and B = benomyl treatment. Unlabelled circles represent other treatments included in the same experiment but not reported in this thesis.

4.2.3.6 Harvest

After six months growth, samples were harvested. Shoots were removed, and rhizomes soaked in water to loosen soil from roots. Roots were then washed and dried and removed from rhizomes. Clean shoots, roots, and rhizomes were weighed (fresh weight), and then dried in an oven at 100 °C for two days, and weighed again (dry weight). Dried leaf samples were homogenized, then %C and % N were measured by combustion on a Leco CN2000, whereas Ca %, K %, Mg %, Na %, P %, S %, B µg/g, Cu µg/g, Fe µg/g, Mn µg/g, and Zn µg/g, were quantified by inductively coupled plasma atomic emission spectroscopy, after microwave digestion in nitric acid. Subsamples of fresh roots were stored at - 20 °C, and in 20 % ETOH at 5 °C, for analysis by molecular and staining techniques respectively. Fresh roots were stained to visualize AM fungal structures and to calculate root length colonization by AMF (Grace and Stribley, 1991). Roots were thoroughly washed, covered in 10% KOH, and incubated at 90 °C for 1.5 h. Samples were then washed with deionised water and covered in 2% HCL for 5 min. Roots were immersed in aniline blue stain (0.05% in 70% glycerol) and incubated at 90 °C for 10 min. Stained roots were covered in acidified glycerol (50 % glycerol, 0.02 % HCL). Root length colonisation was estimated in each sample, using 10 fields of view from 10 roots, using methods previously described (Brundrett, *et al.*, 1996).

4.2.3.7 Statistical analysis

All statistical analysis was carried out using Excel. Comparisons between control and AMF treatments were analysed using TTESTs.

4.2.4 Experiment 3. AMF Inoculation experiment

To measure the impacts of AMF inoculation on *M. giganteus* growth and nutrition, plants were grown with a mixed AMF inoculum in a sterile sand and terragreen medium. Experimental work was carried out in New Energy Farm's commercial research and development glasshouse, in Leamington, Ontario (Canada), in September 2013.

4.2.4.1 Media preparation

Sand and terragreen were mixed in equal volumes (50/50 v/v), and moistened to 25 % with ¼ strength Rorison's nutrient solution. The sand-terragreen mixture (S/T) was then autoclaved twice, twenty-four hours apart.

4.2.4.2 AMF Inoculation preparation

Inoculum was prepared by collecting roots and media from eleven AMF spore cultures growing on *Plantago*, which were then cut up and mixed thoroughly. The inoculation mixture was created using an AMF spore culture collection, held at The University of Warwick. The species of AMF in the inoculation mixture were: *Diversispora spurcum* (AJ276077), *Rhizophagus manihotis* (Y17648), *R. intraradices* (X58725), *Funneliformis mosseae* (AJ306438), *Aculaspora spinosa* (Z14004), *Paraglomus occultum* (AJ276081), *Scutellospora pellucida* (AY035663), *Funneliformis geosporus* (AJ245637), *Claroideoglomus etunicatum* (X17639), *Archaeospora trappei* (Y17634), *Glomus versiforme* (X86687). Sterile inoculum was made by autoclaving inoculation mixture twice, twenty-four hours apart. A background microbe solution was made by soaking 40 g of inoculation mixture in 40 ml of water followed by filtration through a 38 µm sieve, to retain bacteria and remove AMF spores from the wash (Koide and Li, 1989). S/T medium was remoistened to 25 % prior to treatment application.

4.2.4.3 Experimental design

Three treatments were applied to 3.15 kg of S/T growing media 1) control, no AMF or background inoculum, 2) 40 ml of background microbe solution and 88 g of autoclaved inoculum, and 3) 88 g of AMF inoculum. There were 16 replicates for each treatment, a total of 48 plants, and in each pot there was 200 g of medium. Newly emerged rhizomes were cut from two year old micro-propagated rhizomes of *M. giganteus*, which were propagated by New Energy Farms, Leamington, Ontario, research facility (<http://www.newenergyfarms.com/solmass.php>). Roots were removed, and then rhizomes were washed and weighed. All rhizomes were trimmed to a standard weight of 0.6 g (± 0.1 g). One mini rhizome, with one visible bud, was planted into each pot. Treatments and replicates were organised into an augmented Latin square, in blocks of 4 replicates, separated from other treatments by a row in the tray, to avoid cross contamination (see **Figure 4.3**). Plants were watered daily, and fertilised weekly with $\frac{1}{4}$ strength Rorison's nutrient solution.

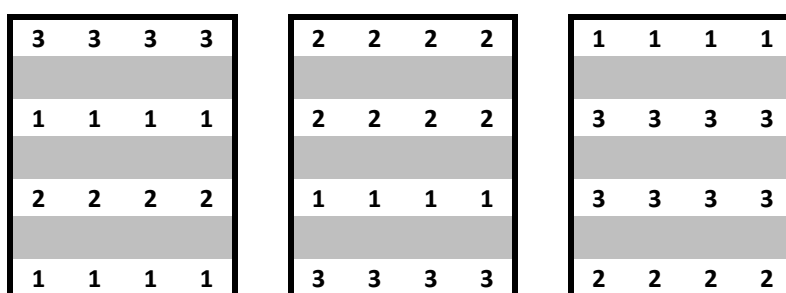


Figure 4.3 Augmented Latin square, four blocks of four replicates with three treatments: 1) control, 2) background microbes, 3) AMF inoculation.

After 6 weeks growth, shoot, and root fresh weights and dry weights were measured. Fresh roots were stained to visualize AM fungal structures and to calculate root length colonization by AMF using the methods in **4.2.3.6 Harvest**.

4.2.4.4 Statistical analysis

All statistical analysis was carried out using Genstat. Comparisons between control, background microbes, and AMF treatments were analysed using multivariate ANOVAs

4.3 Results

4.3.1 Experiment 1. The experimental determination of the phytotoxicity of benomyl to *M. giganteus*

4.3.1.1 Biomass

There was large variability within treatments (**Figure 4.4**), particularly as 38 % of shoots and 65 % roots did not grow (**Figure 4.5**), which skewed the data to a non-normal distribution. When data was log-transformed, the rhizome weights from the 15 mg/kg benomyl treatment were a significantly lower weight than expected (ANOVA $P = 0.04$ *). This treatment was therefore removed from all further analysis. There was no significant effect of benomyl concentration on root, shoot, or rhizome biomass, in either raw data or log-transformed data rhizome DW ($P = 0.499$) FW ($P = 0.409$), roots DW ($P = 0.746$) FW ($P = 0.667$), and shoots DW ($P = 0.763$) FW ($P = 0.379$) (**Appendix Table 4.2a.** and **4.2b.**).

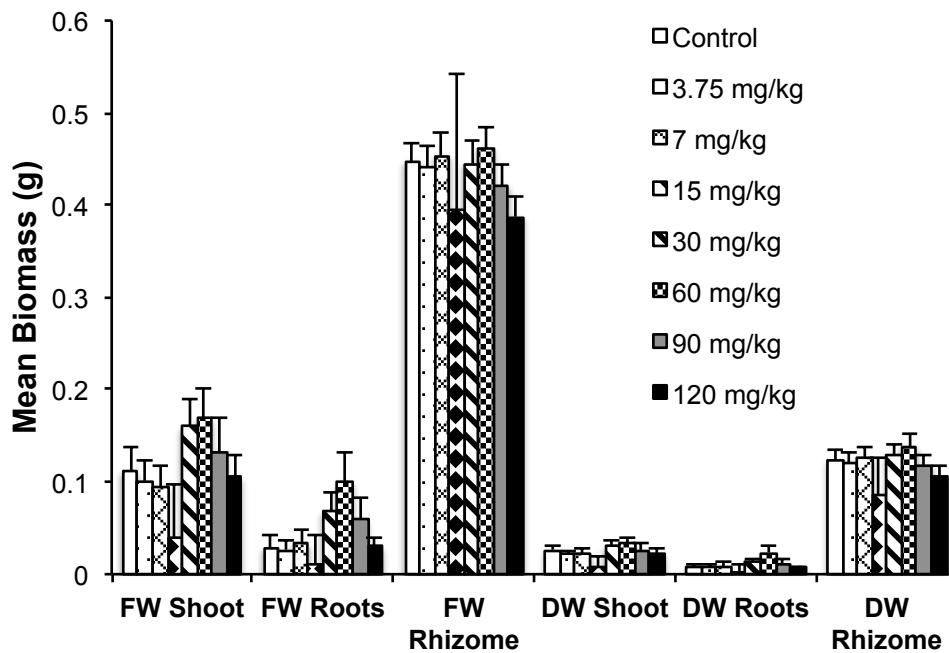


Figure 4.4 Mean biomass of shoot, roots, and rhizomes of *M. giganteus*, grown for 10 weeks with benomyl concentrations ranging between 0 and 120 mg/kg soil). Error bars represent \pm SEM, $n = 32$ for each of the eight treatments.

4.3.1.2 Viable growth

Shoot growth occurred in a total of 159 samples (62 %), while root growth was detected in 88 samples (35 %) (**Figure 4.5, Figure 4.6**). An increase in benomyl concentration was significantly correlated with a decrease in the number of emerged shoots (slope = -0.067, $r = -0.941$, $P = 0.002$ *) (**Figure 4.5**). There was no significant correlation between benomyl concentration and the number of samples with emerged roots (slope = 0.018, $r = 0.449$, $P = 0.264$) (**Figure 4.5**).

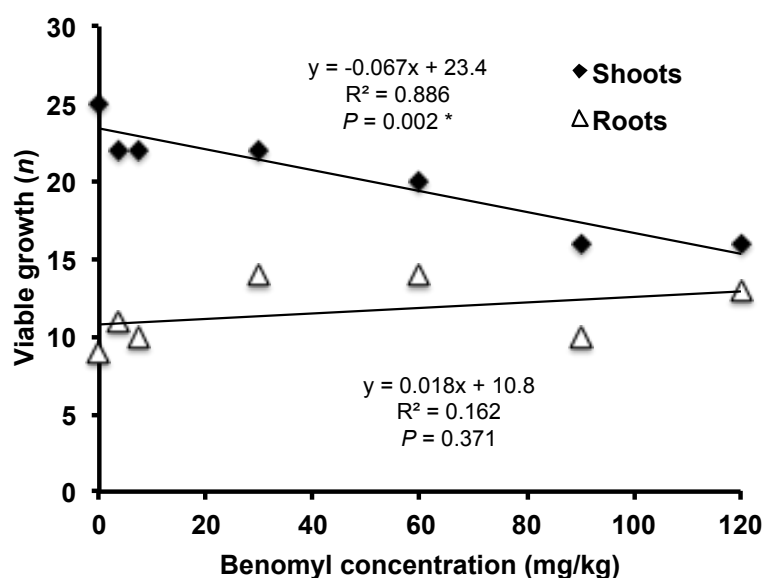


Figure 4.5 Number of samples (n) where viable growth was observed, shoots (black diamonds) and roots (white triangles), with increasing levels of benomyl fungicide (0 - 120 mg/kg), out of the 32 replicate rhizomes planted. The trend lines show linear regressions, and the equation, R^2 and P values, for shoots (top) and roots (bottom), where * = $P < 0.05$. Analysis includes all samples excluding anomalous treatment 15 mg/kg sample.

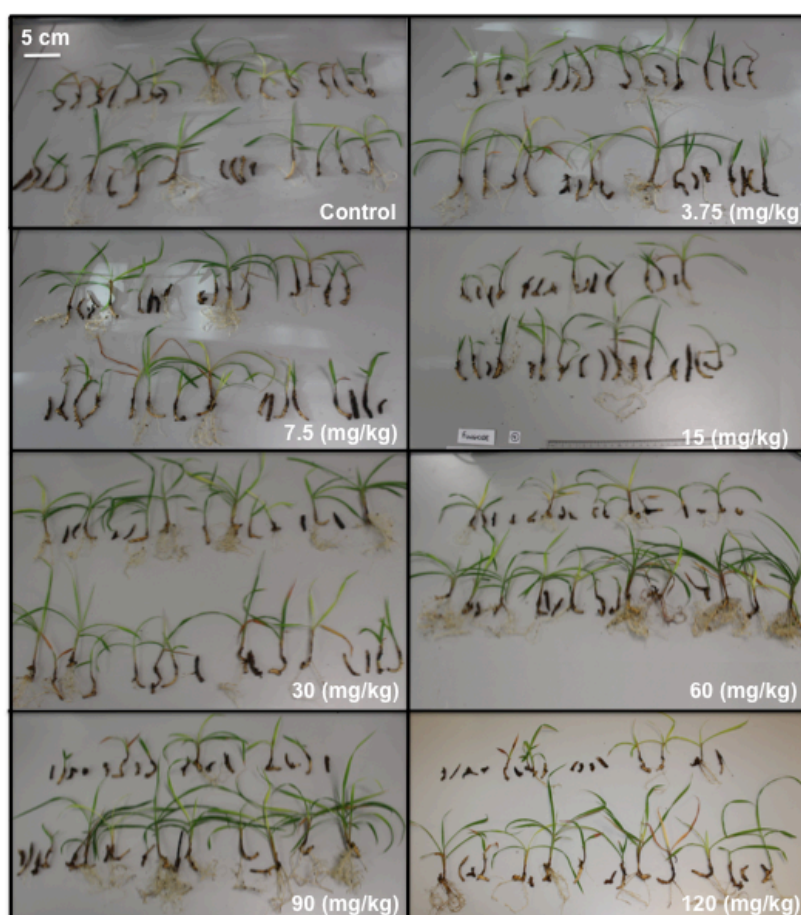


Figure 4.6 All samples from the trial after ten weeks growth, with increasing levels of benomyl application from 0 to 120 (mg/kg). Scale bar = 5 cm.

4.3.1.3 Determining the concentration of benomyl to use in AMF reduction experiment, to determine the importance of AMF to *Miscanthus* growth and nutrition

There was no significant effect of benomyl on *M. giganteus* growth (**Appendix Table 4.2**), however there was a noticeable trend for decreasing biomass as benomyl concentration increased. The highest shoot, root, and rhizome biomass was at 60 mg/kg soil, and appeared to decrease at 90 and 120 mg/kg (**Figure 4.4**). Therefore, the highest dose, which showed no sign of phytotoxicity was 60 mg/kg. The use of this concentration is supported in the literature review (**Appendix Table 4.1**), as 60 mg/kg is in the range of optimum reduction (40 - 60 mg/kg reduced RLC by 73.1 %), and was near the mean concentration used in fifty-eight studies analysed (68.4 %), and all studies at 53.3 mg/kg eliminated colonisation when benomyl was applied to soil before seeds were sown.

4.3.2 Experiment 2. The reduction of AMF using benomyl fungicide

4.3.2.1 AMF colonisation

Benomyl applied as a soil drench (60 mg/kg), significantly reduced (TTEST $P < 0.001$) mean (\pm SEM) AMF root length colonisation from 23.3 (\pm 2.9) % in the control, to 2.8 (\pm 0.6) %, at eleven weeks (**Figure 4.7**). After six months, some AMF colonisation had reappeared and was not quite significantly reduced (TTEST $P = 0.56$), with 46.3 (\pm 11.8) % RLC in controls, and 17.8 (\pm 2.4) % RLC in benomyl treatments (**Figure 4.7**).

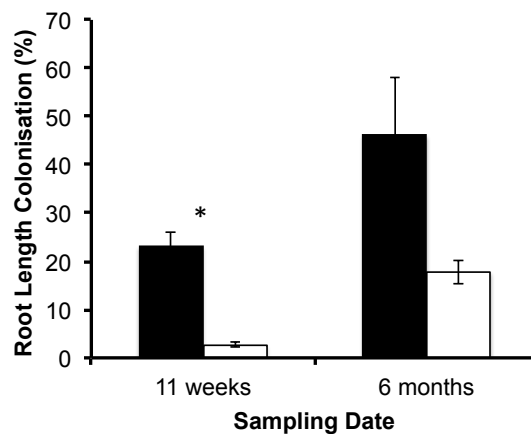


Figure 4.7 AMF root length colonisation of *M. giganteus* after 11 weeks and 6 months growth, in the benomyl soil drench (white bars) and control (black bars) treatments. Error bars represent \pm SEM, $n = 4$, * = TTEST $P < 0.05$ of AMF root length colonisation between control and fungicide treatment.

4.3.2.2 Plant Growth

Only four of the five replicates grew for each treatment. Growth of *M. giganteus* in the benomyl treatment was lower than in the control throughout the experiment, with a mean (\pm SEM) of 37.8 (\pm 2.5) % shorter stem length per pot, 39.0 (\pm 1.4) % fewer leaves per pot (**Figure 4.8**), and 37.5 (\pm 1.5) % fewer shoots per pot. However, there were no significant differences in stem length, numbers of leaves, or numbers of shoots between treatments at any stage of growth (TTEST $P > 0.05$).

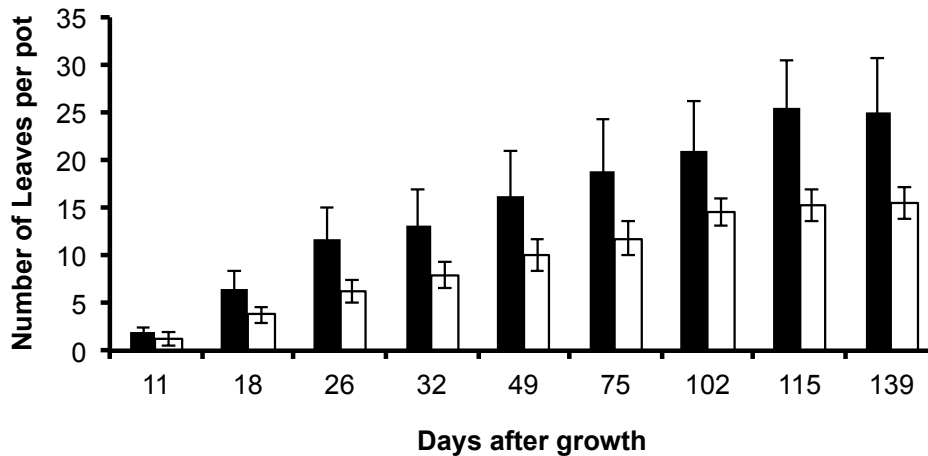


Figure 4.8 Mean number of leaves per pot in control (black bars) and benomyl treated (white bars) *M. giganteus*, from 11 to 139 days growth, where error bars represent \pm SEM, $n = 4$.

4.3.2.3 Photosynthesis

Overall, the photosynthetic rate of control plants was $18.7 (\pm 0.39 \text{ SEM})$ and for benomyl plants $17.9 (\pm 0.35 \text{ SEM})$, the difference was not significant (TTEST $P = 0.199$). Control leaves had significantly higher photosynthesis (11.6 %) on the first day of measurements (TTEST $P = 0.029$ *) (**Figure 4.9**). However, photosynthesis on other days was not significantly different.

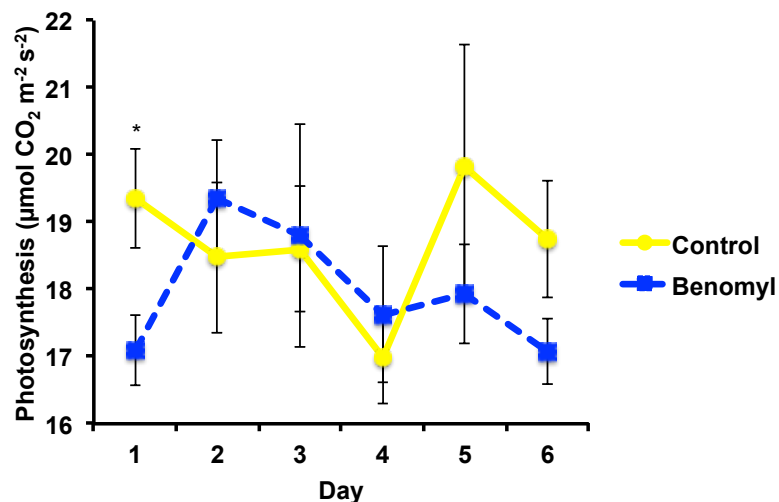


Figure 4.9 Photosynthesis (PN) $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ of *M. giganteus*, in control (yellow circles), and benomyl treated leaves (blue squares), where error bars = \pm standard error of the mean, $n = 4$, and * = TTEST $P < 0.05$ between control and benomyl photosynthetic rates on each day.

4.3.2.4 Biomass

The biomass of *M. giganteus* in the benomyl treatment was significantly lower than the control. Dry weight of the benomyl treatment was lighter than control, in shoots by 38.3 % (TTEST $P = 0.032$ *), roots by 34.8 % (TTEST $P = 0.065$), and rhizomes by 44.3 % (TTEST $P = 0.031$ *) (**Figure 4.10**, **Figure 4.11**, **Figure 12**). **Figure 4.11** illustrates the large difference in rhizome size between treatments, and **Figure 4.12** shows that controls were bigger, bushier, and greener than benomyl treated plants, after two months.

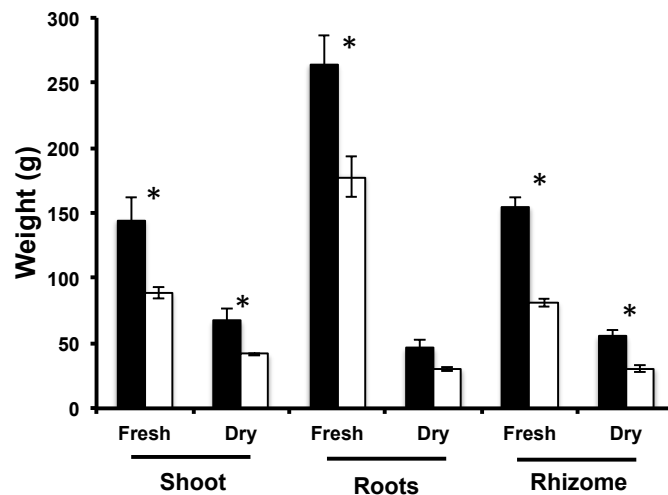


Figure 4.10 Fresh and dry weights of shoots, roots, and rhizomes, after 6 months growth with benomyl (white bars) and control (black bars), where error bars = +/-standard error of the mean, $n = 4$, and * = TTEST $P < 0.05$ between control and benomyl weight.



Figure 4.11 Rhizomes in control (a) and with benomyl (b) treatments, after six months growth.



Figure 4.12 *M. giganteus* control (left) and benomyl treatments (right), after 2 months growth.

4.3.2.5 Leaf nutrition

Ca and Mg were significantly higher in control leaves, by 17.2 % (TTEST $P = 0.026$) and 26.1 % (TTEST $P = 0.003$), respectively (**Figure 4.13**). There were no significant differences in the remaining leaf nutrients measured between control and benomyl treated plants (TTESTs); % C ($P = 0.066$), % N ($P = 0.139$), % K ($P = 0.782$), % Na ($P = 0.200$), % P ($P = 0.269$), % S ($P = 0.089$), B $\mu\text{g/g}$ ($P = 0.156$), Cu $\mu\text{g/g}$ ($P = 0.994$), Fe $\mu\text{g/g}$ ($P = 0.164$), Mn $\mu\text{g/g}$ ($P = 0.701$), and Zn $\mu\text{g/g}$ ($P = 0.815$).

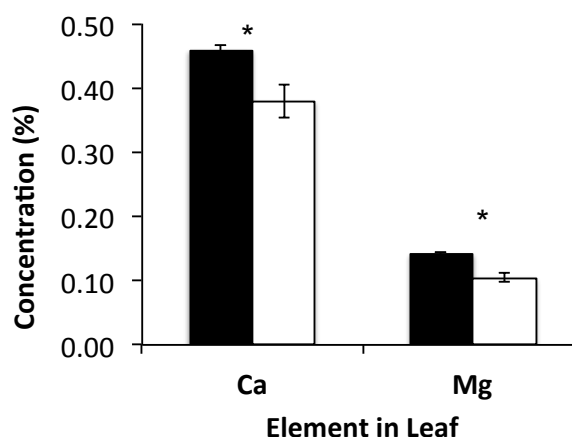


Figure 4.13 Concentration of leaf elements in control (closed bars), and benomyl (open bars) treatments, where error bars = +/- standard error of the mean, $n = 4$, and * = TTEST $P < 0.05$ between control and benomyl treatment concentration of leaf nutrients.

4.3.3 Experiment 3. AMF Inoculation experiment

4.3.3.1 AMF colonisation

The control and background microbe treatments exhibited no AMF colonisation, whereas the AMF inoculation treatment had 6 – 37 % root length colonisation (RLC) (**Figure 4.14**). However, only three rhizomes produced roots in the inoculation treatment (**Figure 4.15**), and there were very few arbuscules, with mainly spores and hyphal growth observed.

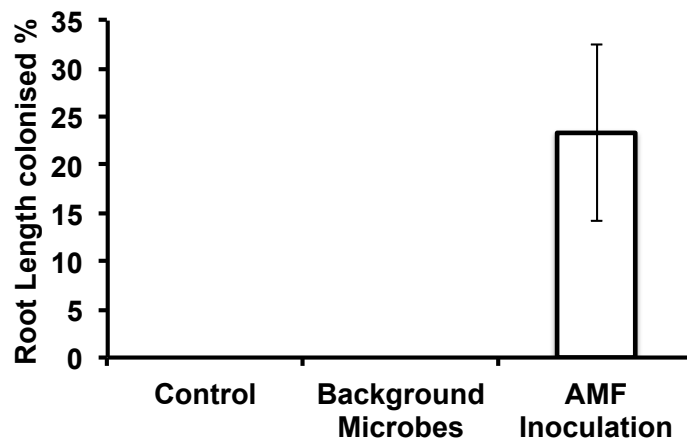


Figure 4.14 Root length colonised in Control ($n = 10$), Background microbes ($n = 6$), and AMF Inoculation ($n = 3$) treatments, error bars represent \pm SEM.

4.3.3.2 Viable growth

From the forty-eight rhizomes planted, the number of viable shoots that grew was thirty-two (67 %), and there was even less viable root growth, with only nineteen rhizomes growing roots (39 %) (**Figure 4.15**, **Figure 4.16**). Viable growth differed between treatments, with the control treatment exhibiting the highest, and AMF inoculation with the least, number of viable plants. From a potential sixteen rhizomes in each treatment, shoot growth occurred in thirteen control plugs (81 %), twelve background microbe plugs (75 %), and seven AMF inoculation plugs (36 %). Root growth success was even lower than shoot growth, occurring in ten control plugs (63 %), six background microbes plants (38 %), and three of the AMF inoculated plants (19 %) (**Figure 4.15**). The trend appears to be that the introduction of greater microbial complexity reduced viability of plant growth.

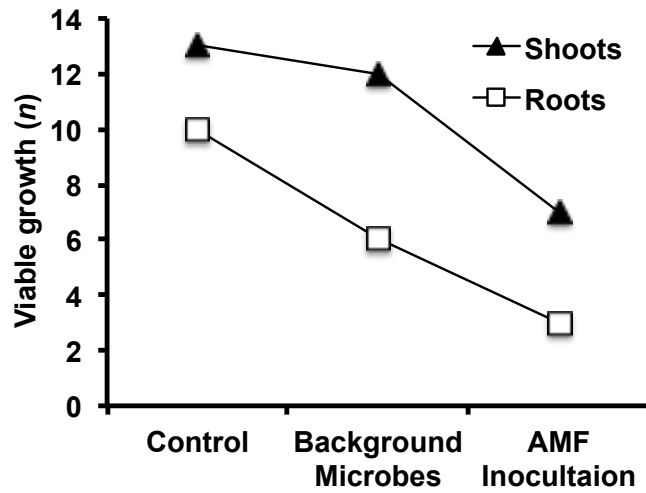


Figure 4.15 Number of samples (*n*), where viable growth occurred in control, background microbes, and AMF inoculation treatments, out of the 16 replicate rhizomes planted in each treatment.

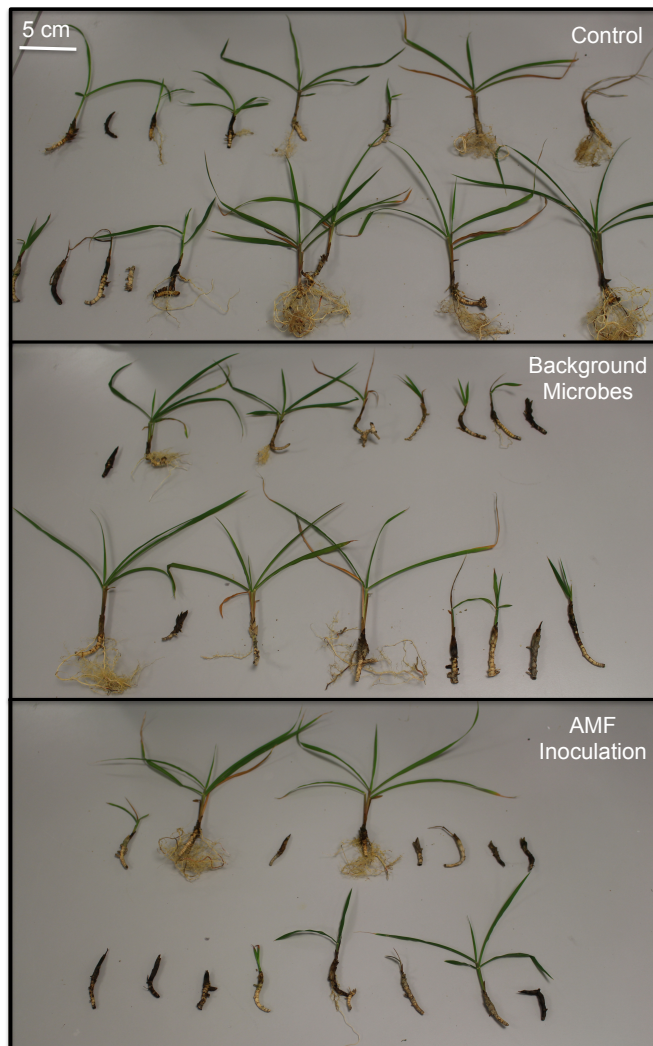


Figure 4.16 All samples from the trial after ten weeks growth, from control, background microbes, and AMF inoculation treatments. Scale bar = 5 cm.

4.3.3.3 Biomass

There was no significant difference in biomass between control and AMF inoculation treatments (**Figure 4.17a**, **Table 4.3a**). There was a higher than expected difference between rows ($P < 0.001$), which means there was a block effect. When the block variability was removed, there was no significant difference between treatments (**Table 4.3a**). A separate analysis was carried out with viable shoot growth only, and there was still no significant difference between the treatments. However when missing values were ignored the shoot and root biomass means increased to weights more similar to controls (**Figure 4.17b**).

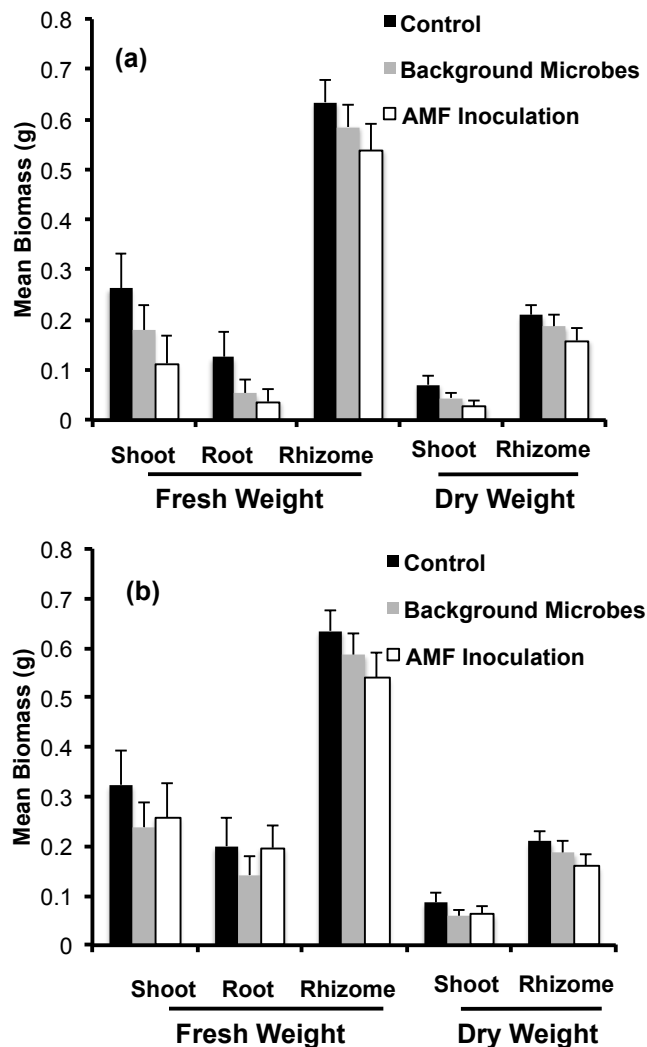


Figure 4.17 Mean biomass of shoot, roots, and rhizomes of *M. giganteus*, after 10 weeks growth: control (black bars), control & microbes (grey bars) and AMF inoculation (white bars). All data points ($n = 48$), including data where no growth occurred (a), and excluding data where no growth occurred (b), where rhizome $n = 48$, root $n = 19$, shoot $n = 32$. Error bars represent \pm SEM.

Table 4.3 Analysis of Variance (ANOVA) of dry weight (DW) and fresh weight (FW), of rhizomes, roots and shoots, testing for variance between treatments accounting for row structure (row stratum), and variance between treatments correcting for row and column variability. (a) all data, including samples where no growth occurred, and (b) viable growth only, samples where no growth occurred have been ignored. Where d.f. = degrees of freedom, v. r. = variance ration, F. pr = Test statistic, * = $P < 0.05$.

(a)						(b)					
Variable	Analysis of variance		d.f.	v.r.	F pr.	Variable	Analysis of variance		d.f.	v.r.	F pr.
DW_Rhizome	Row stratum	Treatment	2	19.5	<.001 *	DW_Rhizome	Row stratum	Treatment	2	19.5	<.001 *
		Residual	9	0.49				Residual	9	0.49	
	Column.row stratum	Treatment	2	0.39	0.681		Column.row stratum	Treatment	2	0.39	0.681
		Residual	31					Residual	31		
DW_Shoot	Row stratum	Treatment	2	43.2	<.001 *	DW_Shoot	Row stratum	Treatment	2	36.6	<.001 *
		Residual	9	0.35				Residual	9	0.62	
	Column.row stratum	Treatment	2	0.75	0.48		Column.row stratum	Treatment	2	1.82	0.196
		Residual	31					Residual	15		
FW_Rhizome	Row stratum	Treatment	2	3.09	0.095	FW_Rhizome	Row stratum	Treatment	2	3.09	0.095
		Residual	9	0.77				Residual	9	0.77	
	Column.row stratum	Treatment	2	0.54	0.586		Column.row stratum	Treatment	2	0.54	0.586
		Residual	31					Residual	31		
FW_Roots	Row stratum	Treatment	2	16.7	<.001 *	FW_Roots	Row stratum	Treatment	2	2.32	0.168
		Residual	9	0.64				Residual	7	1.93	
	Column.row stratum	Treatment	2	0.45	0.643		Column.row stratum	Treatment	2	0.49	0.643
		Residual	31					Residual	4		
FW_Shoot	Row stratum	Treatment	2	31.2	<.001 *	FW_Shoot	Row stratum	Treatment	2	24.3	<.001 *
		Residual	9	0.4				Residual	9	0.99	
	Column.row stratum	Treatment	2	0.51	0.603		Column.row stratum	Treatment	2	2.9	0.086
		Residual	31					Residual	15		

4.4 Discussion

Field populations of fungi improved *M. giganteus* biomass by more than a third, and significantly enhanced Mg and Ca uptake. Whereas, the addition of a multi-species AMF inoculum poorly colonised roots, and had no growth effect on *M. giganteus*.

4.4.1 Experiment 1. The experimental determination of the phytotoxicity of benomyl to *M. giganteus*

The addition of benomyl had no significant phytotoxic effects on *M. giganteus* growth **Figure 4**. However there was large variability in *M. giganteus* growth, even though they were clonally propagated and are genetically identical. There may have been other factors more important to rhizome growth than addition of benomyl, such as epigenetic factors, which explains some of the natural variation in Arabidopsis (Vaughn, *et al.*, 2007), or consistency of watering and sunlight due to position in the glasshouse. These effects should have been

minimised with the design of the experiment, i.e. there were a large number of replicates ($n = 32$), rhizomes weight was standardised, rhizomes were randomised before plantation, and pots were organised into a block structure in the glasshouse. With such large variability, it is difficult to conclude anything with great certainty, however there was a trend, which may be of note. Peak biomass was achieved in the 60 mg/kg benomyl treatment, and there was a decline in biomass at higher concentrations, of 90 mg/kg and 120 mg/kg, which is most evident in **Figure 4.6**. Therefore, the use of benomyl at 60 mg/kg concentration on *M. giganteus* should not have any phytotoxic effects.

The other main issue with the experiment was that different starting materials were used in the two trials. The growing substrate in **1. The experimental determination of the phytotoxicity of benomyl to *M. giganteus*** was AgroMix, a vermiculite, peat, and perlite compost, whereas in experiment **2. The reduction of AMF using benomyl fungicide**, a sandy loam field soil was used. The two substrates are likely to have different potentials to sorb benomyl, which may have affected relative phytotoxicity. However it was important to use compost, to avoid confounding effects of benomyl on soil microbiota. Fungicide application would have altered soil microbiota, which would have been an additional factor to consider if field soil was used, as it is difficult to sterilize soil completely without altering its biochemical structure (Trevors, 1996), and the aim of the experiment was to assess the phytotoxicity of benomyl to *M. giganteus*, not soil microbiota. However, there did not appear to be any phytotoxic effect (**Figure 4.6**), and there was no significant growth decline associated with adding benomyl at 60 mg/kg to *M. giganteus* growth, and this was supported by the use of similar concentrations in literature (**Appendix Table 4.1**).

4.4.2 Experiment 2. Reduction of AMF using benomyl fungicide

Field populations of AMF and other fungi were beneficial for *Miscanthus* growth, compared to benomyl reduced fungi. Fungal populations may have been directly beneficial as mutualistic symbionts, such as mycorrhizal fungi. Other fungi may have been indirectly beneficial, for example saprophytes releasing nutrients (Hattenschwiler, *et al.*, 2005) and maintaining soil aggregation and moisture (Six, *et al.*, 2006). Suppressing the beneficial fungi with benomyl may have decreased rhizosphere functioning and caused the decrease in plant growth.

Many other fungi which typically inhabit the rhizosphere are pathogens, for example the obligate parasite *Olpidium brassicae* builds up in *Brassica napus* roots over time, particularly when there are no crop rotations, and causes yield decline (Hilton, *et al.*, 2013). The suppression of parasites by benomyl would have a positive effect on plant growth, rather than the detrimental effect which was seen. However, some fungi are resistant to benomyl, including some Zygomycota (Summerbell, 1988), and the suppression of most fungi could have decreased competition, allowing opportunist parasites, and benomyl resistant fungi, to flourish. The dominant fungal species sequenced on the roots (Chapter 1) was a *Mortierella* sp., in the Zygomycota family, which is sometimes found to be resistant to benomyl at low doses (Summerbell, 1988), and therefore it may have flourished as a saprophyte with less competition, altering the fungal community structure, rather than eliminating it. Whatever the role of individual organisms, it is clear that lowering field populations of fungi was not beneficial for *M. giganteus* growth. Many other studies, which have used benomyl to lower root colonization by AMF (see **Appendix Table 4.1**), have also altered other fungal populations. Therefore, in these studies too, it is questionable whether the detrimental effect of using benomyl on plant growth was due to suppressing AMF alone, or due to the combined effect of losing a rich fungal diversity in the rhizosphere.

4.4.3 The importance of AMF to *M. giganteus* nutrition

Benomyl reduced Mg and Ca concentration in leaves, which suggests that soil fungi enhanced plant nutrition. Calcium is vital for intracellular membrane transport, and as a component of the middle lamellae. Ca is also thought to be essential for AMF colonisation, as Ca^{2+} spikes are involved in the recognition of endosymbiotic microbes within the common symbiotic signaling pathway (CSSP) (Hepper and Oshea, 1984; Russo, *et al.*, 2013). Mg is the central atom of chlorophyll, and both Ca and Mg stabilise membranes, therefore they are both vital for normal cellular functioning and photosynthesis. However, many soils are Mg deficient, which is thought to be caused by soil acidification, which decreases availability of Mg and increases availability of Al, which in turn interferes with Mg uptake (Ulrich, 1994). AMF inoculation was found to improve Mg, P and N content in salt stressed *Sesbania aegyptiaca*, and reduced Na uptake, thereby alleviating salt stress (Giri and Mukerji, 2004). However, both studies used tree species. Fitter and Nichols (1988) found benomyl application

reduced AMF colonisation of *Trifolium pretense*, which decreased P, Mg, Zn, and Cu flow into the plant. However, there is no direct evidence for Mg and Ca uptake by AMF, and this would be an interesting topic to study further.

Even though Mg and Ca are beneficial for plant growth and nutrition, it is questionable to recommend Mg fertilisation for agricultural practices, as Mg application reduced AMF colonisation of *Allium cepa* and *Ipomoea batatas* (Jarstfer, *et al.*, 1998), but improved AMF colonisation and growth in *Zea mays* (Gryndler, *et al.*, 1992). These mixed results suggest there may be an optimum level of Mg to benefit both partners, and that Mg may be involved in symbiotic interactions. The interactions between AMF and plants are complex, but one of the major factors influencing this relationship is soil fertility. As mentioned earlier, in general, AMF at low nutrient levels can improve growth (Blanke, *et al.*, 2011; Daleo, *et al.*, 2008; Hoeksema, *et al.*, 2010). However, in highly fertilized soil AMF may decrease plant growth (Daleo, *et al.*, 2008). This may be due to the high C cost associated with symbionts, which can take ~ 20 % of newly fixed plant C (Bago, *et al.*, 2000; Vandenkoornhuyse, *et al.*, 2007). Kiers, *et al.* (2011) hypothesised that the AMF-host relationship is based on reciprocal rewards, whereby plants only reward fungal partners which provide benefits, and in return fungal partners only transfer nutrients to hosts which provide carbon rewards. *M. giganteus* may have been Mg and Ca limited, and the symbionts or commensals in the rhizosphere enhanced the availability and plant uptake of these nutrients in the non-fungicide treatment.

4.4.4 Experiment 3. AMF Inoculation experiment

In the inoculation experiment, the AM fungi mainly grew as hyphae, and formed few arbuscules, which suggests that little beneficial nutrient exchange occurred. One explanation could be that the rhizomes had sufficient nutrition, from stored resources or experimental fertilization, and therefore did not enter into the AMF mutualism. It is well supported in the literature that availability of P and N impacts the relationship between AMF and the host plant. Two meta-analyses have shown AMF are more beneficial in P-limited and N-limited soils (Hoeksema, *et al.*, 2010; Lekberg and Koide, 2005).

The soil nutritional status has also been shown to be more important than composition of AMF for enhancing plant nutritional status and growth

(Doubkova, *et al.*, 2013). Fiorilli, *et al.* (2013) found that in *Rhizophagus irregularis* arbuscules, expression of the phosphate transporter *GintPT* halved at a very high concentration of Pi (320 μ M) relative to a lower Pi concentration (32 μ M), whereas the expression of host plant *Medicago trunculata* phosphate transporters *MtPT4* more than tripled at the higher Pi concentration. Therefore, in higher P soils, plants take up their own P, and AMF down regulate their high affinity P transporters. However, Gosling, *et al.* (2013) found that at high levels of P, AMF community structure shifted to half the species richness, which suggests P does effect the relationship of some AMF with plant hosts. However some AMF could continue to colonise roots, and take C from the host, even in high P soil. Perhaps the mutualistic relationship is sustained by providing a different benefit in fertilised soils, such as for other limiting nutrients such as Mg, N, K, Zn and Ca (Marschner and Dell, 1994), or providing disease resistance through the common mycelial network (Jung, *et al.*, 2012).

Another explanation for the presence of AMF in fertilised soils, may be they exhibit no benefit at all to the plant, and actually represent a carbon drain. AMF have been found to develop parasitism induced during development, by the environment, or even genetically (Johnson, *et al.*, 1997). Some endophytes are able to switch lifestyle from being a mutualistic symbiont to a parasite. For example *Epichloe festucae*, an endophyte of *Lolium perenne* is able to make the switch by a single gene mutation, which is often linked to an imbalance in nutrient exchange (Kogel, *et al.*, 2006). Therefore, the presence of hyphae, but lack of arbuscules in roots, may explain why there was a decline in viable growth in AMF inoculation treatments. There was no significant difference between control and inoculation treatments, but there was a trend of growth decline with the addition of background microbes and AMF (**Figure 4.15, 4.16, and 4.17**), however the variability was too great to cause a significant effect.

4.4.5 Conclusions

Field populations of fungi improved plant growth more than an AMF inoculum mixture. This indicates that the diversity of fungi from the field was more beneficial to rhizosphere function, plant nutrition and growth, in comparison to the mixed cultures of AMF. This could have arisen from an inability of AMF to grow in the artificial substrate. Alternatively, perhaps if the inoculum was made from AMF spores from the field we could get a better idea of the relative

importance of field populations of AMF, rather than the cultures available, which have an altered lifestyle in culture compared living in the environment, or not be compatible with *M. giganteus*. However, creating spore cultures from environmental samples is very challenging, as current spore extraction methods yield very low numbers of viable spores, and AMF spores need to be cultured and bulked on living plant hosts, as obligate symbionts, which would require a long time period to establish. However, as mentioned previously, plant nutritional status seems to be a more important factor in the improvement of plant growth with AMF inoculation, than AMF identity (Hoeksema, *et al.*, 2010). Therefore, AMF may simply be of little importance for *M. giganteus* growth, in comparison to other soil fungi, including saprophytes (Allen, *et al.*, 2003).

Chapter 5. Diurnal patterns of plant and microbial transcription in the rhizosphere

5.1 Introduction

5.1.1 The circadian clock in plants

Plants undergo daily cycles to adapt to the dramatic change from day to night. Around 30% of plant genes expressed are under control of the circadian clock (Hoffman, *et al.*, 2010; Izawa, *et al.*, 2011; Michael, *et al.*, 2008; Nagasaka, *et al.*, 2009). The circadian clock is a gene regulation network entrained by blue and red light receptors (Schaffer, *et al.*, 2001). Cascades of oscillating genes anticipate dusk and dawn, enhancing metabolic efficiency in light and dark periods. Many of the genes involved with circadian clock regulation either peak in the morning, including *Circadian Clock Associated (CCA1)*, *Late Elongated Hypocotyl (LHY)*, *Pseudoresponse regulators (PRR9 and PRR7)*, or the evening, including *Timing of CAB Expression (TOC1)*, *PRR3*, *Gigantea (GI)*, *LUX Arrythmo (LUX)*, and *Early Flowering Genes (ELF3 and ELF4)* (**Figure 5.1**) (Hsu and Harmer, 2014). The clock is mainly controlled by gene regulation and light, but a number of other inputs have also been shown to adjust the clock, including sucrose (Dalchau, *et al.*, 2011), iron homeostasis (Salome, *et al.*, 2013), and plant defense molecules such as glucosinates (Kerwin, *et al.*, 2011). A wide range of metabolic processes are affected directly or indirectly by the circadian clock, including photosynthesis, carbon metabolism (Haydon, *et al.*, 2011), growth, and development (Farre, 2012). Fixed carbon which accumulates over the photosynthetic period, is converted to starch for storage, and is remobilised during the night for growth and metabolism (Blasing, *et al.*, 2005). The regulation of starch release is tightly controlled, and by dawn, 95 % of starch is consumed, which is thought to be regulated by *CCA1* and *LHY* (Graf, *et al.*, 2010). Circadian regulation also maximises the efficiency of abiotic and biotic stress. An important example of responses to abiotic stress, is the anticipation of cold temperatures, which leads to transcription of cold-related genes (COR) in the day, to enhance freeze tolerance during the night (Eriksson and Webb, 2011). Circadian regulation can also be affected by abiotic stresses. For example, in response to a range of abiotic stresses, *CCA1* and *LHY* were the highest scoring regulatory factors, in a set of genes identified as Multiple Stress Regulators (MSTR) (Kant, *et al.*, 2008). Furthermore, *CCA1* and *LHY*

mutant plants were sensitive to heat, salt stress, and osmotic stress (Kant, *et al.*, 2008). Biotic stresses, such as defense against pathogens, are also thought to be under clock regulation. In plant leaves, *CCA1* regulates the enhanced resistance against downy mildew (Wang, *et al.*, 2011b) and *Pseudomonas syringae* (Bhardwaj, *et al.*, 2011; Ingle and Roden, 2014), in the morning, when infection is most likely. Therefore, the plant circadian clock is a complex system, adjusted by a range of external stimuli, to regulate internal gene networks, to optimise a wide range of metabolic, growth, and defense processes. The vast majority of circadian experiments have been carried out in controlled indoor systems, and very few in natural conditions, where environmental variables are more complex and changeable. One field study showed that 75 % of rice leaf transcripts were found to be diurnally expressed in a paddy field (Izawa, *et al.*, 2011). However the vast majority of studies on plant circadian cycles have analysed processes in the phyllosphere, and their relevance in the rhizosphere is unclear.

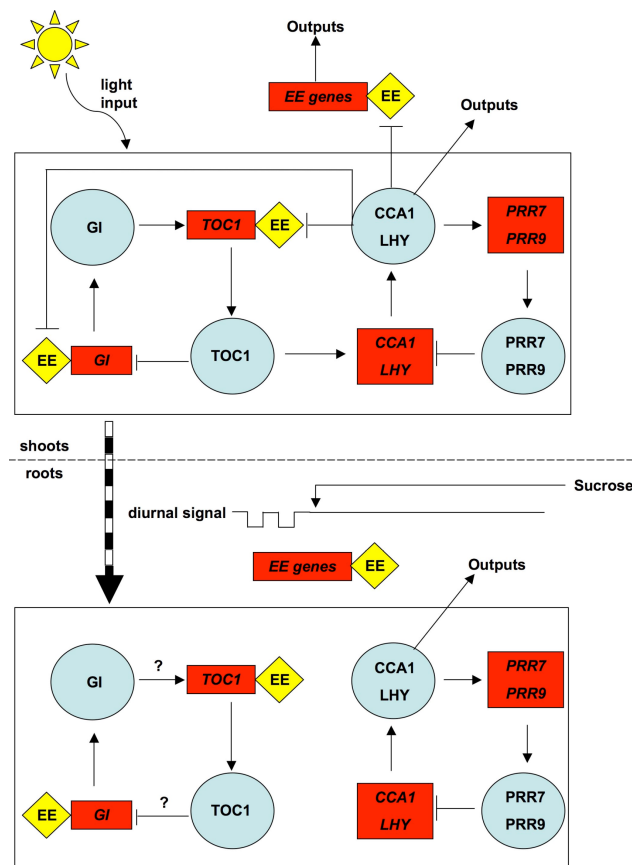


Figure 5.1 A theoretical model of the circadian clock regulation in shoot and roots (James, *et al.*, 2008). EE – evening elements (*LUX*, *ELF3* and *ELF4*), circles – proteins, triangles – genes. *CCA1* and *LHY* suppress *EE* and activate *PRR9* and *PRR7*. *TOC1*, *PRR7* and *PRR9* repress *CCA1* and *LHY*. *GI* promotes *TOC1*, which in turn represses *GI*. Many external stimuli entrain the shoot and root clocks, including light and sucrose, and there is communication between the shoot and root clocks (striped arrow).

5.1.2 Circadian patterns in roots

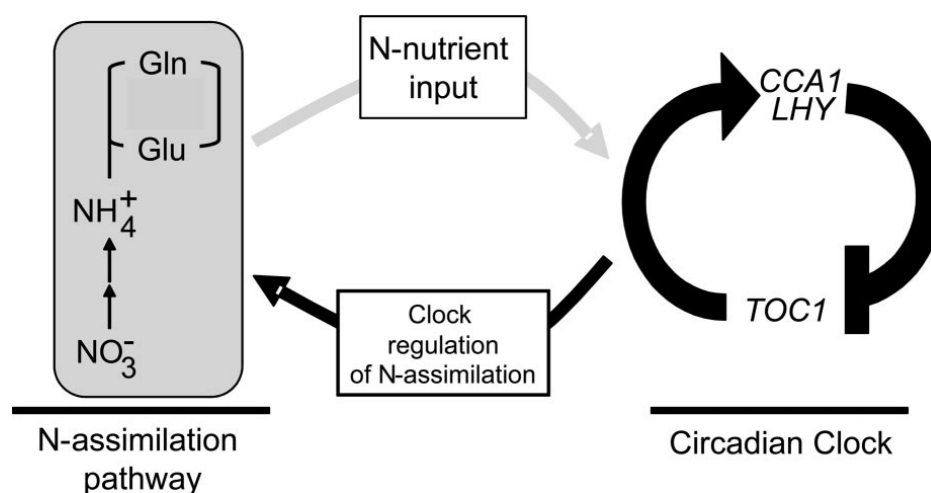
Plant root activity is also affected by circadian clock regulation, but to a lesser degree, and it is much less understood compared to plant shoot circadian control (**Figure 5.1**) (James, *et al.*, 2008). Similar to the shoot clock, many of the oscillating circadian transcription factors in the roots peak in the morning or evening, to bring about cascades of transcription, to anticipate and cope with the change in environmental conditions between night and day. However, James, *et al.* (2008), found only *CCA1*, *LHY*, *PRR7* and *PRR9* regulators were oscillating in circadian rhythms in the roots, whereas other regulators of the shoot circadian clock such as *TOC1* were not, indicating that the circadian clock system is organ specific.

It is thought the root circadian clock could be strongly influenced by sugars entering the roots. Sap flows through stems increasingly throughout the photosynthetic period (Grossiord, *et al.*, 2012), and into the roots and rhizosphere microbes, including arbuscular mycorrhizal fungi (AMF) (Leake, *et al.*, 2006). The accumulative diurnal transport of sap to the roots is thought to lead to a peak in root respiration, and root growth, at dusk (Ruts, *et al.*, 2012). Photosynthetic rates strongly influence rhizosphere respiration, as newly fixed carbon is a limiting factor for growth and respiration (Kuzyakov and Cheng, 2001). That means environmental conditions which affect photosynthesis, i.e. light, temperature, wind speed, stomatal conductance, and air and soil moisture, will affect the rate of carbon fixation, and therefore sugar available for night time growth and respiration, which thereby affects circadian regulation and diurnal processes.

In addition, root hydraulics (Sack and Holbrook, 2006), expression of aquaporins (Clarkson, *et al.*, 2000), and drought stress genes (Marcolino-Gomes, *et al.*, 2014), are under circadian regulation. In turn, drought stress and water availability affect the expression of circadian clock genes (Marcolino-Gomes, *et al.*, 2014). However, root hydraulic activity, and the expression of aquaporins, are also rapidly affected by water availability, irradiance, temperature, transpiration, and even concentration of N, P, and S in the incoming nutrient solution (Carvajal, *et al.*, 1996; Chaumont and Tyerman, 2014; Sack and Holbrook, 2006). For example, it takes 4 - 24 hours to recover aquaporin expression back up to normal activity after nutrient stress is relieved (Carvajal,

et al., 1996). Nutrient uptake has also been linked to circadian regulation. In *A. thaliana*, Mg deficiency altered the expression of circadian clock genes (Hermans, *et al.*, 2010), while *CCA1* is a regulator of N assimilation, and the input of N can adjust the circadian clock (**Figure 5.2.**) (Gutierrez, *et al.*, 2008). These studies indicate that nutrient stress and input could entrain the root clock, and assimilation of nutrients by roots could be circadian regulated, particularly for nitrogen (**Figure 5.2.**).

Figure 5.2 Theoretical model of the interaction between N-assimilation and the Arabidopsis plant circadian clock (Gutierrez, *et al.*, 2008). It was proposed that clock function affects N-assimilation, in part by *CCA1* regulation. In turn, downstream N metabolites, such as Glu and Gln, influence clock function.



5.1.3 Microbial and fungal diurnal patterns

Certain microorganisms also have circadian clocks, including photosynthetic bacteria, in which ~ 30 - 60% of the transcriptome is under circadian regulation (Brown, *et al.*, 2012; Ito, *et al.*, 2009). Some fungi have diurnal patterns. Circadian rhythms in *Neurospora crassa* were first recorded in 1959 (Pittendrigh, *et al.*, 1959), and since then *N. crassa* has been the model organism for studying circadian clocks in fungi. *N. crassa* has precise rhythms of growth, switching between mycelial and conidial development, with conidial growth only occurring at the end of the subjective day (Baker, *et al.*, 2012). Other fungi have been documented to show diurnal patterns, including *Hymenoscyphus pseudoalbidus*, which releases ascospores mostly between 6 - 8 am (Timmermann, *et al.*, 2011). Recently, a Soil Ecosystem Observatory recorded that Arbuscular Mycorrhizal Fungi (AMF) grow in diurnal patterns (Hernandez and Allen, 2013). AMF hyphal productivity, growth and dieback

activities, were greatest between 18:00-23:59 h, and lowest between 06:00-11.59 h (Hernandez and Allen, 2013). However, many studies of fungal daily cycles could be attributed to environmental variables, such as light, wind, and temperature.

5.1.4 Circadian regulation in fungi

A number of genes have been identified which control fungal circadian clocks, to anticipate dusk and dawn. Fungal circadian clock genes identified include Frequency (*FRQ*), White Collar Gene (*WC-1* and *WC-2*), and Vivid (*VVD*), in the model organism *N. crassa* (Collett, *et al.*, 2002; Liu, *et al.*, 1997; Lombardi and Brody, 2005). The molecular components oscillate daily, through a complex negative feedback loop of around 24 hours (**Figure 5.3**). **Figure 5.3** shows many of the components which are activated by light, including *wc-1* and *wc-2*, to transcribe *WC-1* and *WC-2*, which act together to drive the expression of *FRQ*. *FRQ* in turn, represses the expression of *WC-1* and *WC-2* (Collett, *et al.*, 2002). *FRQ* is most unstable at the end of the subjective night, and stops the repression of *frq* mRNA, which is lowest at this point. *frq* mRNA then increases until around midday, when increasing *FRQ* protein negatively regulates *frq* expression (Dunlap and Loros, 2006). Fungal circadian clock genes are thought to be widely conserved (Salichos and Rokas, 2010). Two circadian regulatory genes are found across all fungal lineages, and were therefore probably present in the fungal ancestors; *FWD-1* (F-box/WD-40 repeat containing protein) a protein which degrades *FRQ* (He, *et al.*, 2003), and *FRH* (*FRQ*-interacting RNA helicase) which regulates the stability and phosphorylation of *FRQ* (Guo, *et al.*, 2010). *WC-1* and *WC-2* homologues are not found in the earlier fungal lineages, Chytridiomycota and Microsporidia, however they are present in most fungal phyla, including Zygomycota, Basidiomycota, and Ascomycota, but were lost in the Ascomycota class Saccharomycetes. *FRQ* only evolved in some Ascomycota groups, including Sordariomycetes, Leotiomyces, and Dothideomycetes, including in *Sordaria macrospora* (Nowrousian, *et al.*, 2010), *Leptosphaeria australiensis*, *Creopus spinulosus* (Lewis, *et al.*, 1997), and *Trichoderma pleuroticola* (Steyaert, *et al.*, 2010).

How do fungi in the soil train their circadian clocks in the dark, when light is thought to be the main external cue? Temperature is another main stimulus which influences regulation of fungal circadian clock genes (Liu and Bell-

Pedersen, 2006). However, temperatures do not fluctuate in the soil as extremely as above ground. A number of other factors can affect persistence of rhythmicity in growth and conidiation, including amino acids, salt solutions, and sugars, but it is unclear whether they act by affecting the timing mechanism, or the cessation of conidiation (Sargent and Kaltenbo, 1972).

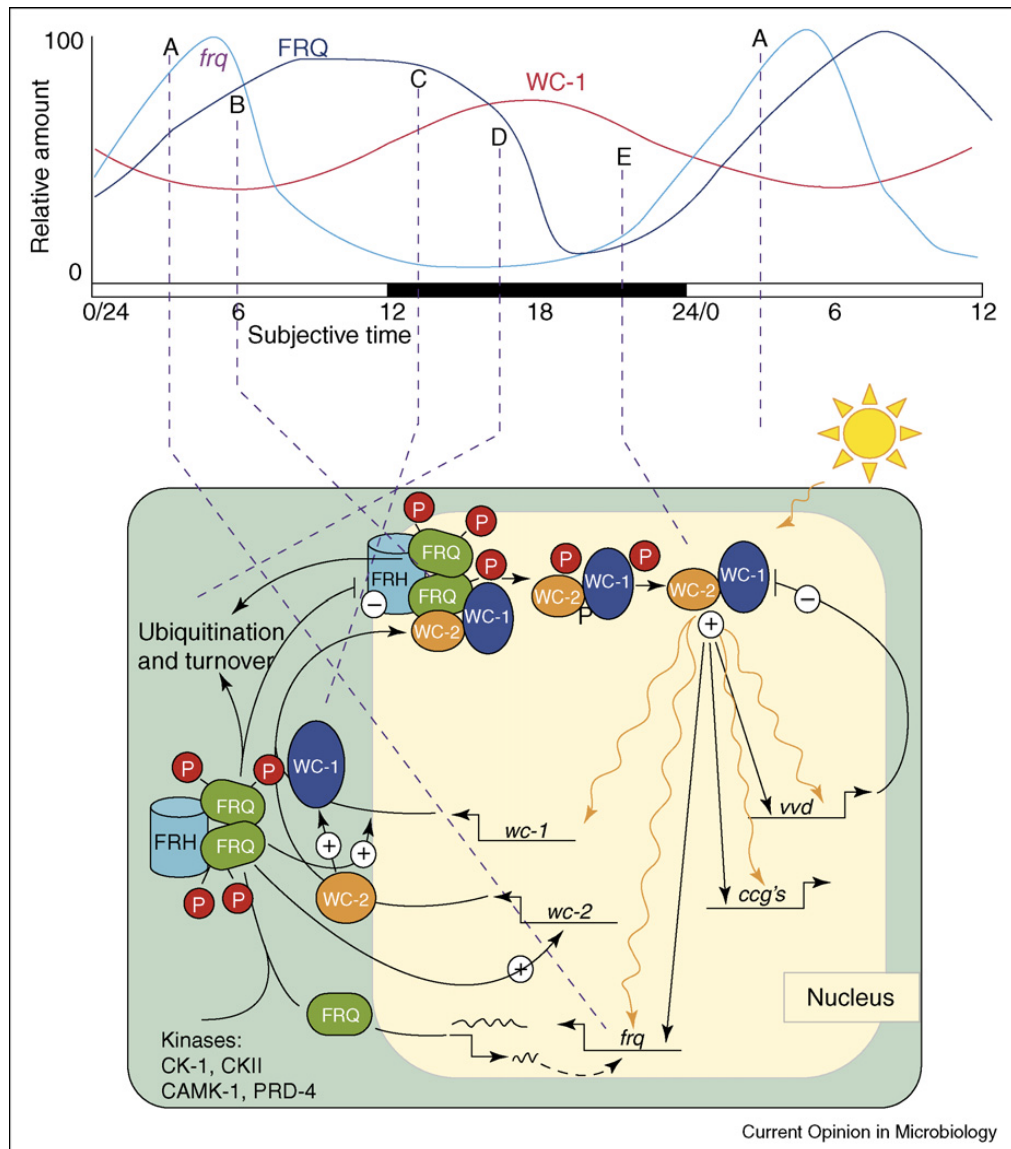


Figure 5.3 The circadian regulatory system in *Neurospora crassa*. Top: diurnal oscillations of *frq* mRNA, FRQ protein, and WC-1 protein in constant darkness, black and white bars represent subjective night and day respectively. Below: Molecular components, where P represents phosphorylation, dotted lines link timing of events, wavy arrows show activation by light, straight arrows indicate positive action, dashed arrows are negative effects (Dunlap and Loros, 2006).

Plant diurnal cycles are well studied, however the response of rhizosphere microbes, in terms of microbial function and activity, to root circadian patterns is unknown. Many plant genes either peak or trough near dusk or dawn, and the

peak and trough of rhizosphere respiration is often near dusk and dawn respectively. Next generation sequencing technologies have opened a window to allow this kind of interaction to be studied for the first time.

5.1.5 Plant and fungal genomes

A key advance is the availability of plant and fungal genomes to use as a reference for transcript annotation. A number of plant genomes have been annotated, including the model plant species *Arabidopsis thaliana* (Zimmermann, *et al.*, 2004), *Oryza sativa* (rice) (Goff, *et al.*, 2002), and *Sorghum bicolor* (Swaminathan, *et al.*, 2010). The genomes of over 50 fungi have also been sequenced and annotated, and many more are being sequenced as part of the 1000 Fungal Genome Project and other initiatives (<http://1000.fungalgenomes.org/home/>). Annotated fungal genomes include the Ascomycota fungi *Saccharomyces cerevisiae* (yeast) (Griffin, *et al.*, 2002; Velculescu, *et al.*, 1997), *Neurospora crassa* (Pomraning, *et al.*, 2011), *Parastagonospora nodorum* (Hane, *et al.*, 2007), the Glomeromycota species *Rhizophagus irregularis* (Tisserant, *et al.*, 2013), and the Basidiomycota species *Coniophora puteana* (Floudas, *et al.*, 2012).

5.1.6 Meta-transcriptomes

Meta-transcriptomic analysis has the potential to simultaneously characterise structure and function of communities, to find out which species are active and what their functional roles are. The understanding of root community function will be vastly improved with the utilisation of meta-transcriptomes, to accumulate all knowledge of microbial function and taxonomy, to find the answers to interesting questions which were unimaginable, too time consuming, and too expensive, before these techniques became available. There are relatively few meta-transcriptomes published (Mason, *et al.*, 2012; Yergeau, *et al.*, 2012; Zaura, 2012), and fewer in the soil (Bailly, *et al.*, 2007; Damon, *et al.*, 2011; de Menezes, *et al.*, 2012; Urich, *et al.*, 2008). There is only one other meta-transcriptome of roots available, however, this was only a taxonomic analysis, as so few functional genes were detected (Turner, *et al.*, 2013), meaning that in reality the study could have been carried out use conventional taxonomic markers. The current study will fully utilize meta-transcriptome technology, to

characterize structure and function of fungal, bacterial, archaeal, and plant transcription simultaneously, and to find out whether plant and fungal circadian clock regulation is present, in root environmental samples.

5.1.7 Hypothesis

The hypothesis of this work, is that rhizosphere fungi, including arbuscular mycorrhizal fungi, have co-adapted to plant root diurnal patterns of gene expression and carbon levels, and their activity and functioning will differ from dusk to dawn. In turn, plant circadian regulation is affected by microbial activity and function, such as nutrient cycling. To test this hypothesis, a comparison was made between morning and evening activity of fungal and AMF communities, and a meta-transcriptome of plant roots and rhizosphere eukaryotes, was made, using the roots of *Miscanthus giganteus* in a bioenergy cropping field, as a model environmental system.

5.2 Methods

5.2.1 Sample Collection

Soil samples were collected from a commercial *M. giganteus* field near Brattleby, Lincolnshire, before trough and peak rhizosphere respiration rates, at 8 am and 8 pm, over three consecutive days, 30th August – 1st September 2012. Samples were taken 25 m from the field edge, within 10 m of GPS location SK 94225 BNG 81115. Random coordinates were generated using Genstat within this 20 m x 20 m area, and samples were taken 15 cm from the base of approximately 2 m tall *M. giganteus* clumps, nearest to the random coordinate. Soil samples of 15 cm x 15 cm x 5 cm were collected, and immediately stored on ice. Within one hour, roots were extracted from the soil, cleaned, cut into 0.5 cm pieces, thoroughly mixed up, and divided into 100 mg portions of roots, which were placed into separate centrifuge tubes. Roots were stored in RNeasy lysis solution at 4 °C overnight, then stored at -20 °C, according to manufacturer's guidelines (Qiagen). White secondary roots were selected for analysis, with dark discoloured roots discarded.

5.2.2 Diurnal rhizosphere respiration estimation

To estimate diurnal changes in rhizosphere activity continuous rhizosphere respiration rates were measured, in the commercial *M. giganteus* crop, by the Centre for Ecology and Hydrology (CEH), Lancaster. Respiration was measured from randomised circular plots, established in 2010 and lying between shoots to avoid damage, with a permanent 2 m diameter PVC chamber collar, as described by Case, *et al.* (2012). Respiration from five replicates was measured with a Li-Cor 8100 closed dynamic chamber (Heinemeyer and McNamara, 2011) using three hourly means from 1st August - 10th September 2011. To estimate rhizosphere respiration, general soil respiration was subtracted from total respiration, using the continuous measurements from control plots (with roots), and root exclusion plots (without roots). Peak and trough times of rhizosphere respiration, and therefore activity, were estimated to be 10 pm and 10 am respectively.

5.2.3 Environmental measurements

Gaseous fluxes and other environmental conditions were measured throughout the experiment, and for more than a year before root sampling. Eddy Covariance (EC) systems, LiCor 7500 open path IRGAs and Gill R3 sonic anemometer, mounted on a flux tower two meters above the crop, measured a number of parameters every thirty seconds, including Gap Filled NEE $\text{mmol m}^{-2} \text{s}^{-1}$ (net exchange of CO_2 in and out of the system), total ecosystem respiration $\text{mmol CO}_2 \text{m}^{-2} \text{s}^{-1}$, and GPP $\text{mmol CO}_2 \text{m}^{-2} \text{s}^{-1}$ (gross primary production or plant C fixation). Data was logged with an Mk4 Hydra data logger (Institute of Hydrology). Meteorological data were provided by a Didcot Instruments AWS with: cup anemometer, wind vane, air and wet bulb temperatures, and a rainguage (Rimco). These data were logged as 30 minute averages, except for the rainfall which was the 30 minute total.

5.2.4 Molecular methods

5.2.4.1 DNA extraction

DNA was extracted from six samples; one biological replicate from 8 am and 8 pm sampling times per sampling location on the three consecutive days. For each extraction, 100 mg of roots was taken out of RNA/later solution, and flash

frozen in liquid nitrogen. Frozen roots were placed in a 2 ml screw top RNase-free centrifuge tube, with one 3 mm tungsten carbide bead, and homogenised in a TissueLyser for 20 seconds at 30 beats/s. DNA was extracted using a QIAGEN FastDNA SPIN Kit for Soil, according to manufacturer's instructions, and stored at -20 °C.

5.2.4.2 RNA extraction

For each extraction, 100 mg of roots was taken out of RNA/later solution, and flash frozen in liquid nitrogen. Frozen roots were placed in a 2 ml screw top RNase-free centrifuge tube, with one 3 mm tungsten carbide bead, and homogenised in a TissueLyser for 20 seconds at 30 beats/s. Lysates were then passed through a QIAshredder spin column (Qiagen), according to the manufacturer's protocol. RNA was then extracted using an RNeasy Mini Kit (Qiagen, Manchester, UK), following the manufacturer's instructions. DNA contamination in the eluted RNA, was digested in solution by adding 2.5 µl DNase 1, 10 µl Buffer RDD (Qiagen), before being made up to 100 µl with RNase free water, and incubated at room temperature for 20 mins. RNA was then purified according to the RNA Cleanup protocol, from the RNeasy Mini Kit manual (Qiagen), and eluted in 30 µl of RNase free water. To identify any DNA contamination, the purified RNA was loaded onto an electrophoresis agarose gel, RNA purity was quantified on a NanoDrop (Thermo Scientific), and a PCR with ITS1F and ITS4 (White, *et al.*, 1990) primers was carried out. Any DNA contaminated samples were re-purified, and checked again. Sample quality and quantity were analysed using a Bioanalyzer (Agilent) and a NanoDrop (Thermo). Purified RNA was then stored at -80 °C until further processing.

5.2.4.3 Reverse Transcription (RT) PCR for amplicon sequencing

Twelve RNA extract samples, two biological replicates each from 8 am and 8 pm on the three consecutive days, were used for Reverse Transcription PCR (RT-PCR). RT-PCR was performed to synthesise DNA from the RNA extracts. Primers were ligated for 5 min at 75 °C, in a reaction mixture of; 4 µl RNase free water, 1 µl random hexamer primer 100 µM (Thermo Scientific), 5 µl RNA, set up on ice. The following reagents were then added, for Reverse-Transcription PCR; 1 µl MegaMix (Microzone), M-MLV 5 x reaction buffer (Promega), 0.4 µl

RNase-inhibitor (20 U/ µl, Roche), 0.8 µl M-MLV reverse transcriptase (200 U/ µl), 3.8 µl RNase free water.

5.2.4.4 AMF PCR for amplicon pyrosequencing

The six DNA extracts, and twelve cDNA extracts, were used for AMF amplicon pyrosequencing. For AMF specific 18S rRNA amplicon analysis, cDNA and DNA was amplified using the primer set AML1 and AML2 (Lee, *et al.*, 2008), with universal tails at 5' ends, for multiplex identification. *Forward* 5'-(**Universal Tail**) **GTGTGAAATTGTTACGCT** – (AML1) ATCAACTTTTCGATGGTAGGTAGGATAGA-3', and *Reverse* 5' (**Universal Tail**) **ACTGGCCGTCGTTACAAC** - (AML2) GAACCCAAACACTTTGGTTTCC-3'. AMF PCR cycle conditions were as follows: 94 °C 5 min, 94 °C 1 min, 40 cycles of 57 °C 1 min, 72 °C 1 min, then 72 °C 10 min, 15 °C ∞.

5.2.4.5 Total fungal community PCR for amplicon pyrosequencing

The six DNA extracts, and twelve cDNA extracts, were used for total fungal community amplicon pyrosequencing. For total fungal community amplicon analysis, cDNA and DNA was amplified using general fungal primers ITS1F and ITS4 (White, *et al.*, 1990), with universal tails at 5' ends, for multiplex identification. *Forward* 5'-(**Universal Tail**) **GTGTGAAATTGTTACGCT** - (ITS1F) – CTTGGTCATTTAGAGGAAGTAA -3', and *Reverse* 5'-(**Universal Tail**) **ACTGGCCGTCGTTACAAC** - (ITS4) TCCTCCGCTTATTGATATGC-3'. Fungal ITS PCR cycle conditions were as follows: 95 °C 3 min, 30 cycles of 95 °C 3 min, 55 °C 1 min, 72 °C 1 min, then 72 °C 10 min, 12 °C ∞.

5.2.4.6 Amplicon pyrosequencing

PCR products, from the reverse transcription, AMF and total fungal community cycles, were purified using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK). Multiplex Identifiers, MID1-8 of the 454Standard MID set, were added to the 5' end of PCR products during a second round of PCR amplification, to identify samples on the multiplex, and hybridise DNA to the capture beads, respectively, according to the 454 Sequencing Guidelines for Experimental Design 2012 handbook (Roche, 454 Life Sciences Corp.,

Branford, USA). Unidirectional sequencing, from the forward primer, was carried out on a Roche 454 GS Junior Titanium by a service provider (MicroPathology, Warwick Science Park, UK), following the manufacturers protocol (Roche, 454 Life Sciences Corp., Branford, USA).

5.2.4.7 Meta-transcriptome sequencing

Twelve RNA extracts were used for meta-transcriptome sequencing, two biological replicates each, from each of the 8 am and 8 pm sampling times over three consecutive days, 30th August – 1st September 2012. Total RNA was sent to The Genomic Analysis Centre (TGAC), Norwich, UK, to prepare and sequence. The quantity and quality of the samples were assessed by TGAC using the Qubit fluorometer (Invitrogen) and 2100 Bioanalyzer (Agilent Technology). An Illumina TruSeq mRNA library was constructed by TGAC using the Illumina TruSeq RNA Sample preparation guide v2 (Illumina Inc.) in accordance with the manufacturer's protocol. In brief, 1 µg of total RNA was purified and enriched for mRNA using poly-T oligonucleotide attached magnetic beads. Two rounds of purification were used. During the second elution of poly-A RNA, the RNA was fragmented and primed for cDNA synthesis. cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Invitrogen) and random primers. Second strand cDNA synthesis was carried out and the DNA was subjected to end repair, "A" tailing and ligation. cDNA templates were enriched by 15 cycles of PCR as per manufacturer's instructions. The amplified library was quantified using LapChip GX using the High Sensitivity DNA chip (PerkinElmer). The library was normalised to 10 nM for generation of sequence clusters on a sequencing flow-cell on the Illumina c-Bot instrument. Sequencing library cluster generation was carried out on a paired-end flow cell on the Illumina cBot (Illumina Inc.) according to the manufacturer's instructions; 1% PhIX Control v3 spike (Illumina Inc.). All sequencing was carried out on an Illumina HiSeq 2000 (Illumina Inc.), with 100 bp paired-end read metric, TruSeq SBS V3 Sequencing kit (Illumina Inc.) and version 1.13.48 RTA. FASTQ files were generated and demultiplexed according to library-specific indices by CASAVA v. 1.8.2.

5.2.5 Bioinformatic analysis

5.2.5.1 Amplicon pyrosequencing bioinformatic analysis

All sequence processing was carried out using QIIME (Caporaso, *et al.*, 2012), default settings, unless otherwise stated. Sequences were truncated at a length suggested by the `quality_scores_plot` function, to remove poor quality 3' ends, and standardise length (ITS = 543 bp, AMF = 384 bp). Sequences were denoised with Acacia (Bragg, *et al.*, 2012), with default settings, except that a minimum average quality threshold of 25 was used. Sequences were dereplicated, and chimeras were removed, using UCHIME (Edgar, *et al.*, 2011). Operational Taxonomic Units (OTUs) were picked with 97 % similarity, using the `uclust` picking method (Edgar, 2010). The cluster seed from each OTU was picked for the representative set. Representative total fungal OTUs were assigned to the UNITE ITS database (Abarenkov, *et al.*, 2010). Representative AMF OTUs were assigned to a database made from clone libraries of AMF 18S rDNA, within the same *M. giganteus* field, and from a range of Glomeromycota 18S sequences from NCBI (Chapter 3). Bray Curtis non-metric multidimensional scaling ordination plots (NMDS), analysis of similarity (ANOSIM), and hierarchical cluster analysis, were carried out on relative abundance OTU tables, using Primer 6 software (Primer-E, Luton, UK). The UNITE database was unable to assign taxonomy to the majority of OTUs, therefore statistical analysis was carried out on both un-assigned OTUs and taxonomically assigned OTUs (either UNITE or AMF clone database).

5.2.5.2 Plant transcriptome bioinformatic analysis

The plant transcriptome analysis was carried out by Yi-Fang Wang and Jonathan Moore, at the University of Warwick, UK. To analyse the plant (*M. giganteus*) transcriptome, the meta-transcriptome was aligned to the *Sorghum bicolor* genome, and then annotated using the *S. bicolor*, *Arabidopsis thaliana*, and rice genome annotations that were downloaded from Phytozome (<http://www.phytozome.net>; (Goodstein, *et al.*, 2012)). The meta-transcriptome was also aligned to a *M. giganteus* transcriptome (Chouvarine, *et al.*, 2012), but this was not used in the analysis as there was low sequence mapping rate. There are currently no *Miscanthus* genomes available. The *S. bicolor* genome (Paterson, *et al.*, 2009) was the closest relative to *Miscanthus* with an annotated

genome and transcriptome, and *S. bicolor* has been shown to be a useful reference genome for Andropogoneae grasses (Swaminathan, *et al.*, 2010).

All raw data passed the FastQC quality checks (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), except the first thirteen basepairs of each sequenced reads, which is a common error in Illumina transcriptomes (Hansen, *et al.*, 2010). The problem was overcome by using the local alignment setting in Bowtie2 (Langmead and Salzberg, 2012), which only partially aligns sequences, to avoid bad quality ends. The HTSeq (Anders, *et al.*, 2014) was applied to show the read counts that mapped to the annotated features, i.e. genes. Around 70 % of pair-end reads were aligned to the *S. bicolor* genome concordantly, and the remaining sequences were removed from analysis.

Statistical analysis was carried out using R. Multivariate analyses were carried out with all mapped reads, including an Analysis of Similarity (ANOSIM), Multidimensional Scaling Plot (MDS), and a Principle Components Analysis (PCA), comparing day and night samples. Gene expression clustering across samples was carried out using the Weighted Correlation Gene Network Analysis (WCGNA) algorithm (Langfelder and Horvath, 2008), and Pearson correlations were calculated between gene expression groups and environmental variables. The environmental variables (for collection of data see methods section '**Environmental measurements**'), included corrected soil heat flux (W m^{-2}), net radiation (W m^{-2}), downward global solar radiation (W m^{-2}), wind speed (m s^{-1}), wind direction degrees, air temperature ($^{\circ}\text{C}$), rainfall (mm), measured VPD (hPa), gap filled sensible heat flux (W m^{-2}), gap filled latent heat flux (W m^{-2}), gap filled NEE ($\mu\text{mol m}^{-2} \text{s}^{-1}$ net exchange of CO_2 in and out of the system), total ecosystem respiration ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ plant and soil respiration), GPP ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ gross primary production, plant C fixation). A heat map of the correlations between expression clusters and environmental variables was then produced.

Annotated counts were normalised with DESeq2 (Anders and Huber, 2010), by adding the pseudocounts to proceed the logarithm transformation i.e. $y = \log_2(n + n_0)$, where n represents the raw counts and n_0 is a positive constant. Normalisation and counts were also performed by using edgeR (Robinson, *et al.*, 2010), by transferring raw counts into \log_2 counts per million (logCPM). In

edgeR, a filter was applied to remove genes without at least one read per million in two of the samples (Anders, *et al.*, 2013). A principle components analysis, and a multidimensional scaling plot, was created using edgeR normalised counts. ADONIS was used, with Bray Curtis dissimilarity of edgeR normalised reads, to test for differences between all morning and evening read profiles, and the differences between morning and evening profiles with interaction between days. Genes which were significantly differently expressed between day and night, Differentially Expressed Genes (DEGs) were found using both DESeq2 and edgeR, of the interaction between day and night and between different days, with the thresholds of adjusted P-value (FDR) < 0.05 and log₂ fold change ratio more than 1 or smaller than -1. Gene expression levels of all DEGs were plotted onto a heat map. Scaled heatmaps were then created, by transformation of gene expression to a normal distribution, using the Z-score $(=(x-\mu)/\sigma)$, where x = the normalised read counts, μ = mean of the normalised read counts, and σ = standard deviation. Then hierarchical clustering (distance = maximum distance, linkage criteria = ward) was applied based on the Z-score, leading to the identification of six clusters. Clusters 5 and 6 had very similar patterns of expression and were combined to be treated as one cluster, 5 & 6. For gene ontology analysis and visualization, all DEGs were uploaded onto AmiGO (Carbon, *et al.*, 2009), using the Gene Ontology (Ashburner, *et al.*, 2000) GO term enrichment tool, and The Arabidopsis Information Resource (TAIR) database for annotation, with a threshold of $P < 0.05$ to identify significantly enriched GO terms within DEGs (Boyle, *et al.*, 2004).

A number of plant circadian clock gene sequences; *CCA1*, *ELF3*, *GI*, *LHY*, *LUX*, *PRR7*, *PRR9*, and *TOC1*, were downloaded from the National Centre for Biotechnology Information (NCBI) online database. The plant homologues of circadian clock genes (plant species and Genbank code) were: *CCA1* (*Arabidopsis thaliana* U28422.1, *Ananas comosus* AY750151.1, *Hordeum vulgare*, JN603242.1), *LHY* (*A. thaliana* NM_001197953.1, *Triticum aestivum* HQ222606.1, *Sorghum bicolor* JQ350844.1 (partial), *Nicotiana attenuata* JQ424913.1, *Phaseolus vulgaris* AJ420902.2), *ELF3* (*A. thaliana* NM_128153.2), *GI* (*A. thaliana* AF076686.1), *LUX* (*A. thaliana* NM_001203093.1, *Hordeum vulgare* KC668274.1 and KC668258.1), *PRR7* (*A. thaliana* NM_120359.2, *Theobroma cacao* XM_007016568.1, *Hordeum vulgare* EU331928.1), *PRR9* (*A. thaliana* AB046953.1, *Castanea sativa* EF694006.1, *Brassica rapa* GU219476.1), and *TOC1* (*A. thaliana* AF272039.1. *Hordeum*

vulgare JQ791235.1, *Glycine max* NM_001248273.1, *Populus tremula* HQ833401.1). The circadian clock genes listed were searched for using the blastn (Altschul, *et al.*, 1997) function on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastp), selecting *S. bicolor* (taxid:4558) as the Organism, and more dissimilar sequences (discontiguous megablast) for the program selection. The *S. bicolor* gene with the lowest E-value was selected as the most likely circadian gene homologue. To visualise relatedness of homologues, the *S. bicolor* circadian gene homologues were aligned to the homologues from the other plant species, listed previously, and plotted on neighbor-joining phylogenetic trees, with the T-coffee 8.93 multiple sequence alignment tool <http://www.ebi.ac.uk/Tools/msa/tcoffee/> (Notredame, *et al.*, 2000). The expression level of each *S. bicolor* circadian gene homologue was then searched for in the transcriptome of each sample, and was plotted onto a heatmap, to visualize expression over time. A normalised heatmap was then created, using Z-scores of gene expression, as described previously. Circadian clock genes which have been identified as either having larger expression in the morning and evening in controlled systems previously, were highlighted on the heatmaps as being morning or evening components, respectively (Hsu and Harmer, 2014; James, *et al.*, 2008).

5.2.5.3 Meta-transcriptome analysis of root eukaryota

The microbial eukaryote meta-transcriptome analysis was carried out by Hyun S. Gweon, at the Centre for Ecology and Hydrology, Wallingford, UK. Unmapped reads, which could not align to the *S. bicolor* genome, were used to analyse the microbial eukaryote microbiome, to reduce the contamination of plant sequences. Quality filtering was carried out using TRIMMOMATIC, which removed adapters, and allowed; maximally two mismatched seeds, paired end reads above quality score 30, and single reads above quality score 10. TRIMMOMATIC then extended and clipped reads, scanned the read with 4-base wide sliding window, cut reads when the average quality per base dropped below 15, and removed reads which were less than 50 bases long after these steps. After quality checking, FRAGENESCAN was used to predict coding regions with the default settings. Subsampling was performed by inflating each unmapped sample to its raw sample size by adding blank reads, then N reads were randomly picked from each inflated sample, where N is the library size of

the smallest raw sample. Three databases were prepared: a) fungi, protists, archaea, bacteria and nematodes; and b) fungi, and c) bacteria and archaea, all from NCBI nr (non-redundant) protein database. Then BLAT (BLAST-like alignment tool <http://www.ncbi.nlm.nih.gov/pubmed/11932250>) was run on the coding DNA sequences against the databases. Where there were hit(s) from both pairs of reads, both reads were merged together. MEGAN5 was used to perform Lowest Common Ancestor (LCA) algorithm on the BLAT result of each read. Function was assigned to reads, using SEED annotation (http://www.theseed.org/wiki/Home_of_the_SEED). Subsampled taxonomy and functional proportions were plotted into bar charts using Qiime (Caporaso, *et al.*, 2012), where all taxa with less than 100 reads, functions with less than thirty reads for SEED bar charts, and the unassigned sequences, were removed from bar charts. Statistical analyses were carried out in R. A number of multivariate analyses were made for taxonomy and function of OTUs, including ADONIS (permutational multivariate analysis of variance using Bray-Curtis distance matrices), SIMPER (similarity percentages), and NMDS (non-metric multidimensional scaling plot), at phyla, class, order, family, genus, and species level for taxonomy, and Level 1 - 4 for SEED functions.

5.2.5.4 Fungal genome alignments

The meta-transcriptome sequences were aligned to the fungal genomes *Rhizophagus irregularis* (Tisserant, *et al.*, 2013), *Parastagonospora nodorum* (Hane, *et al.*, 2007), and *Coniophora puteana* (Floudas, *et al.*, 2012). *Rhizophagus irregularis* is currently the only Glomeromycota genome sequenced, and was used to represent arbuscular mycorrhizal fungi in the roots. *Parastagonospora nodorum* was the top contributor to the differences between morning and evening expression, in the meta-transcriptome, according to the SIMPER analysis. It was also the closest relative with a published genome to *Ophiosphaerella herpotricha*, the most abundant taxa in the ITS pyrosequencing library, which also showed major differences between morning and evening. Both *Parastagonospora nodorum* and *Ophiosphaerella herpotricha*, are part of the Phaeosphaeriaceae family. The Basidiomycota species *Coniophora puteana* was the second top contributor to the differences between morning and evening expression, in the meta-transcriptome, according to the SIMPER analysis.

5.2.5.5 Fungal circadian clock genes in the meta-transcriptomes

A number of fungal circadian clock gene sequences; *FRQ*, *VVD*, *WC-1*, and *WC-2*, *FRH*, and *FWD-1*, were downloaded from the National Centre for Biotechnology Information (NCBI) online database and searched for in the three fungal transcriptomes (*Parastagonospora nodorum*, *Coniophora puteana*, and *Rhizophagus irregularis*) using HMMER (Finn, *et al.*, 2011). The fungal circadian clock genes (fungal species and Genbank code) were: *FRQ* (*Neurospora crassa* U17073.1, *Sordaria macrospora* XM_003351350.1, *Leptosphaeria australiensis* U25851.1, *Creopus spinulosus* U25850.1, *Trichoderma pleuroticola* GQ849327.1, *Phaeosphaeria nodorum* XM_001793595.1, *Cordyceps militaris* XM_006666172.1), *VVD* (*N. crassa* AF338412.1, *Sordaria macrospora* XM_003344804.1, *Verticillium albo-atrum* XM_003001693.1), *WC-1* (*N. crassa* X94300.2, *Phaeosphaeria* sp. Sn23-1 GQ149727.1, *Gibberella moniliformis* HM045019.1, *Agaricus bisporus* XM_006462965.1), and *WC-2* (*Gibberella moniliformis* HM045020.1, *Phaeosphaeria* sp. Sn48-1 GQ254709.1, *N. crassa* Y09119.1, *Schizophyllum commune* XM_003035648.1), *FRH* (*N. crassa* AY818711.1), and *FWD-1* (*N. crassa* AY685231.1). In each fungal transcriptome, a number of homologous scaffolds were found for each circadian clock gene, so all the homologues were aligned and a multiple sequence profile was created to represent the circadian-like homologue. The circadian multiple sequence profiles were then aligned to the database circadian clock genes (described previously), using Clustal Omega (Sievers, *et al.*, 2011), and phylogenetic neighbor-joining trees were made using PhyML (Guindon and Gascuel, 2003). The circadian multiple sequence profiles were also used to make a search for circadian homologues in the meta-transcriptomes, to generate total and subsampled read counts of each homologue in each sample.

5.3 Results

5.3.1 Diurnal respiration Measurements

The net exchange of CO₂ from the system exhibited a diurnal pattern, which was higher during evenings, in the preliminary study (see **Appendix Figure 5.1** for respiration from roots and root-exclusion plots, and **Figure 5.5** for subtracted rhizosphere respiration), and for at least a month either side of sampling, including on sampling days (**Figure 5.6**). At the morning sampling time

respiration was falling rapidly or near trough level, and at the evening sampling time was nearing peak respiration (**Figure 5.6**). Plant carbon fixation (GPP) also had a diurnal cycle on most days, at least a month either side of sampling, which peaked around midday and troughed during the evening, including during the sampling period (**Figure 5.7**). Morning sampling time was at the start of carbon fixation, whereas the evening sampling time was at the end of carbon fixation.

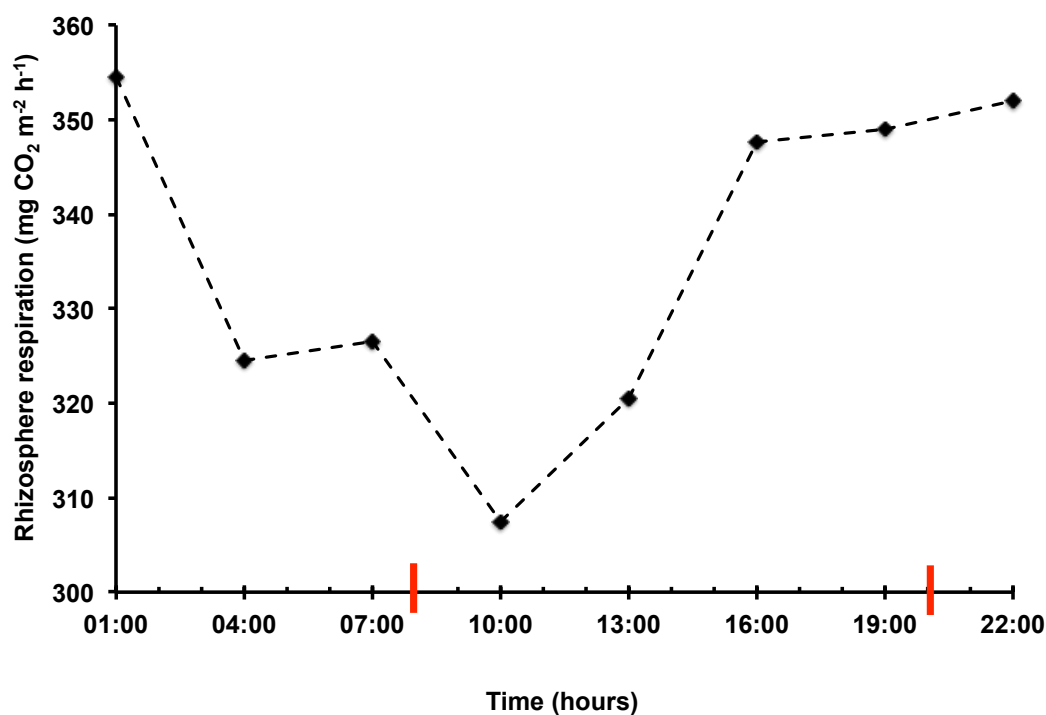


Figure 5.5 Subtracted rhizosphere respiration (mg CO₂ m⁻² h⁻¹), of the three hourly mean from 1st August - 10th September 2011. Red bars are 8 am and 8 pm. Standard deviation was not calculated as this is a subtractive mean (estimation.)

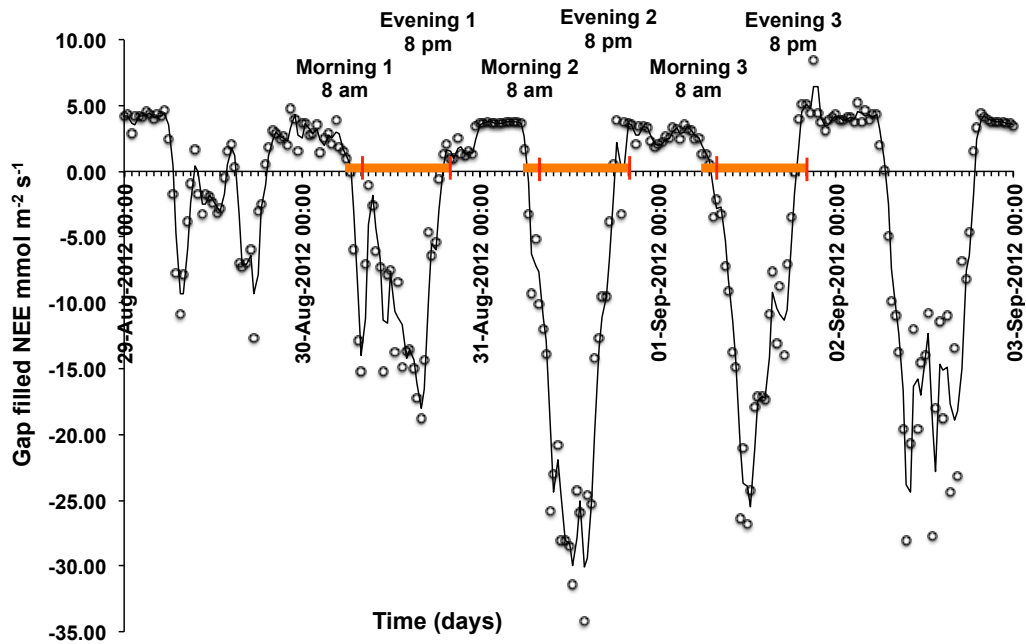


Figure 5.6 Gap filled NEE $\text{mmol m}^{-2} \text{s}^{-1}$, net exchange of CO_2 in and out of the system, which is plant CO_2 uptake minus plant and soil respiration. Orange horizontal bars represent daylight hours, red vertical bars represent sampling times 8 am (mornings) and 8 pm (evenings).

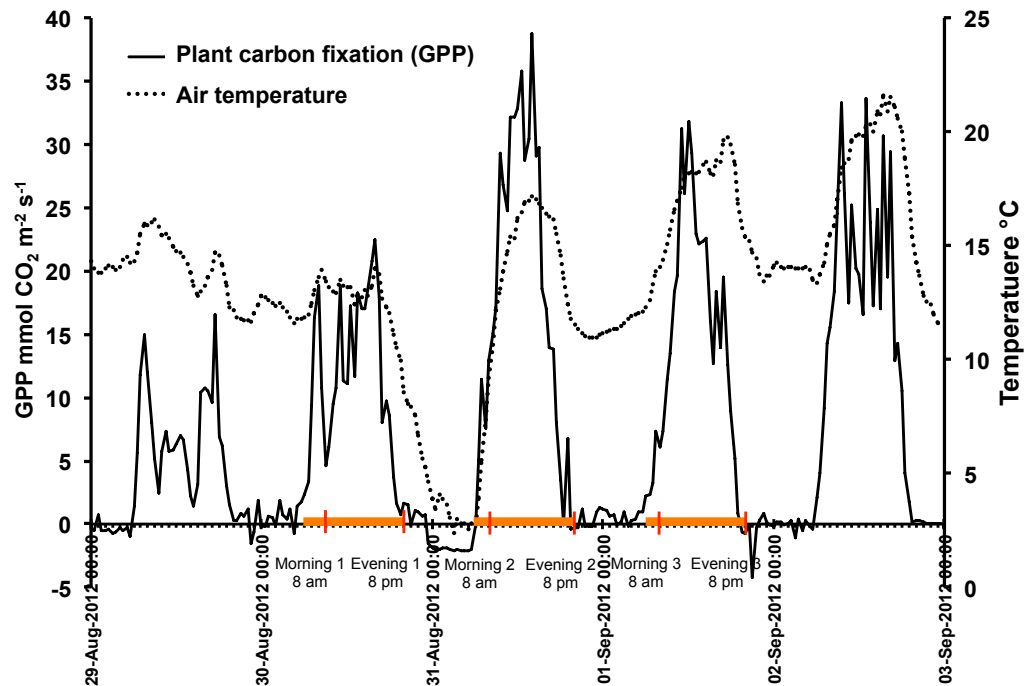


Figure 5.7 Hourly means of plant carbon fixation (GPP $\text{mmol CO}_2 \text{m}^{-2} \text{s}^{-1}$), and air temperature, during sampling times \pm one day. Orange horizontal bars represent daylight hours (sunrise 6 am – sunset 8 pm), and red bars sampling times 8 am (mornings) and 8 pm (evenings).

5.3.2 *Miscanthus giganteus* root transcriptome

5.3.2.1 Transcriptome sequence numbers

There were a total of 181,996,028 paired end reads, from the HiSeq lane. The number of raw reads for each sample was 18,852,622 (Morning 1 Rep 1), 14,557,659 (Morning 1 Rep 2), 9,162,990 (Evening 1 Rep 1), 13,719,054 (Evening 1 Rep 2), 21,757,962 (Morning 2 Rep 1), 20,974,459 (Morning 2 Rep 2), 18,302,113 (Evening 2 Rep 1), 12,750,011 (Evening 2 Rep 2), 14,880,138 (Morning 3 Rep 1), 10,571,336 (Morning 3 Rep 2), 14,561,595 (Evening 3 Rep 1), 11,906,059 (Evening 3 Rep 2) (**Figure 5.8**). Around 70 % of reads were aligned to the *S. bicolor* genome, ~50 % aligned once and ~20 % more than once, and the remaining ~30 % could not be aligned concordantly (**Figure 5.8**). The number of annotated genes before the filter was 27,608, and after the filter there were 21,176.

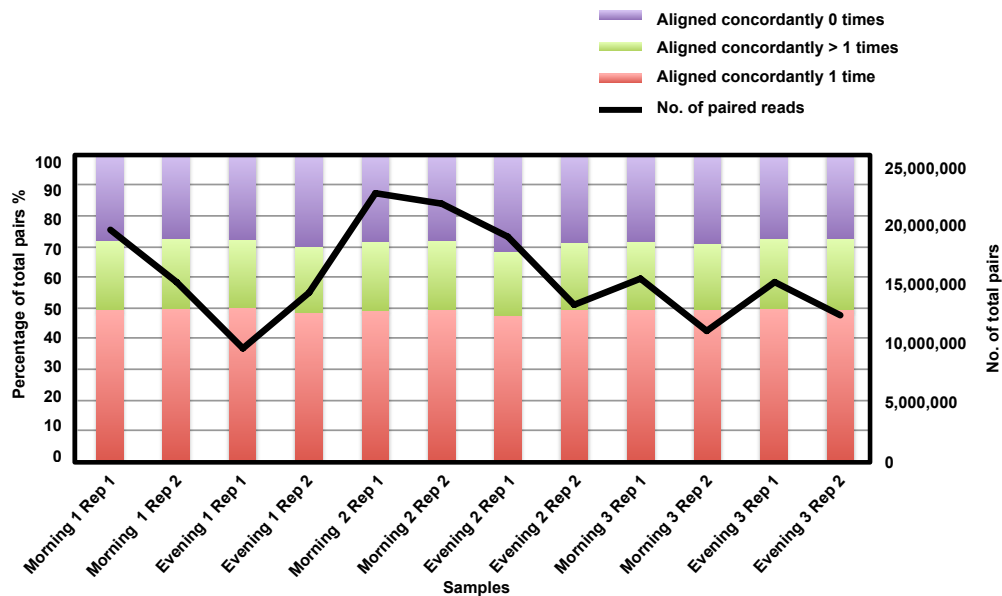


Figure 5.8 Local alignment result of meta-transcriptome to *S. bicolor*, with Bowtie2, where the three consecutive days, 1 – 3 represent the 30th August – 1st September 2012, morning is 8 am samples, and evening is 8 pm samples.

5.3.2.2 Gene expression of morning and evening *M. giganteus* transcriptomes

The dissimilarity between all genes was plotted onto an MDS plot (**Figure 5.9**), and a PCA analysis (**Figure 5.10**), which both show that morning and evening profiles clustered separately, for most sampling times. The difference between evening and morning gene expression profiles was significantly different (ADONIS $r^2 = 0.143$, $P = 0.05$), and the significance level was greater when the interaction between days was included in the model (ADONIS $r^2 = 0.143$, $P = 0.01$). In addition, there were 1,141 significant DEGs in the interaction between morning and evening and different days, from DESeq2 and edgeR normalised sequences (**Figure 5.11**).

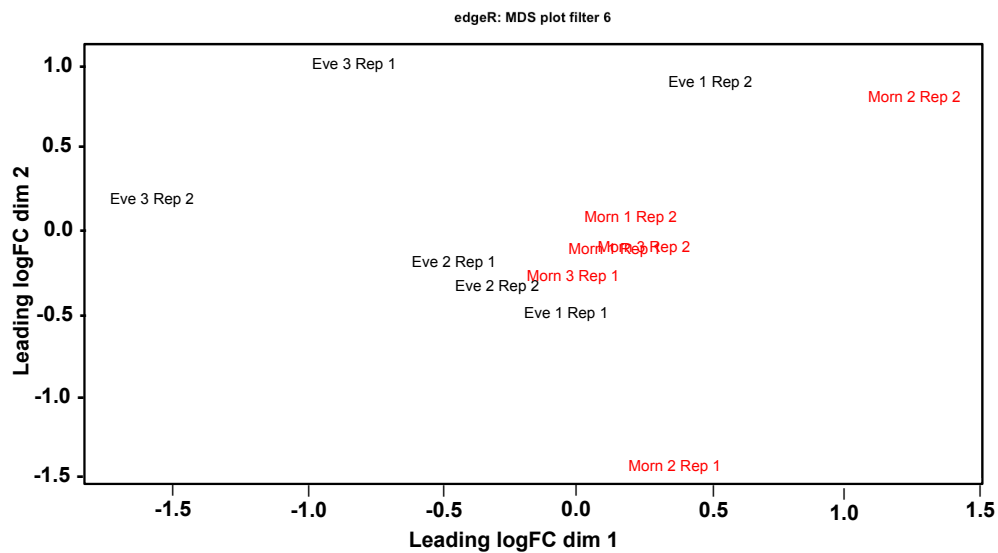


Figure 5.9 MDS plot of edgeR normalised gene counts, with a filter applied, of morning (morn) and evening (eve) samples, replicates (rep) 1 and 2.

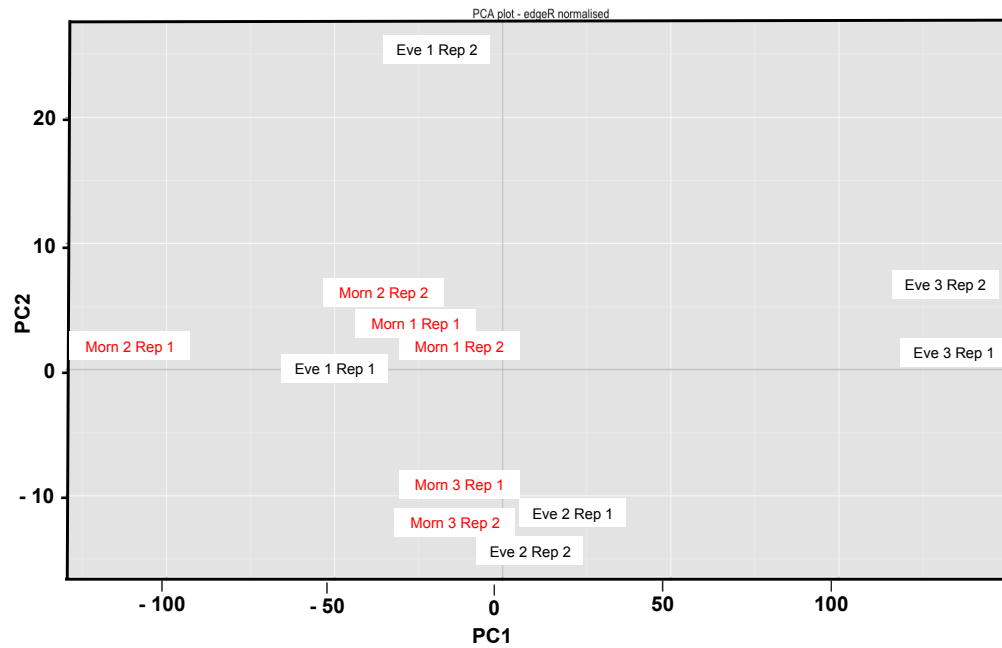


Figure 5.10. PCA plot of edgeR normalised gene counts, with a filter applied, of morning (morn) and evening (eve) samples, replicates (rep) 1 and 2.

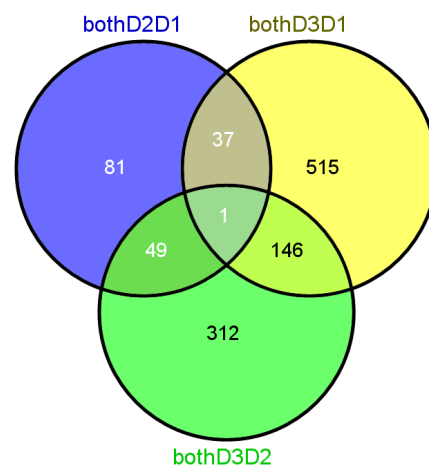
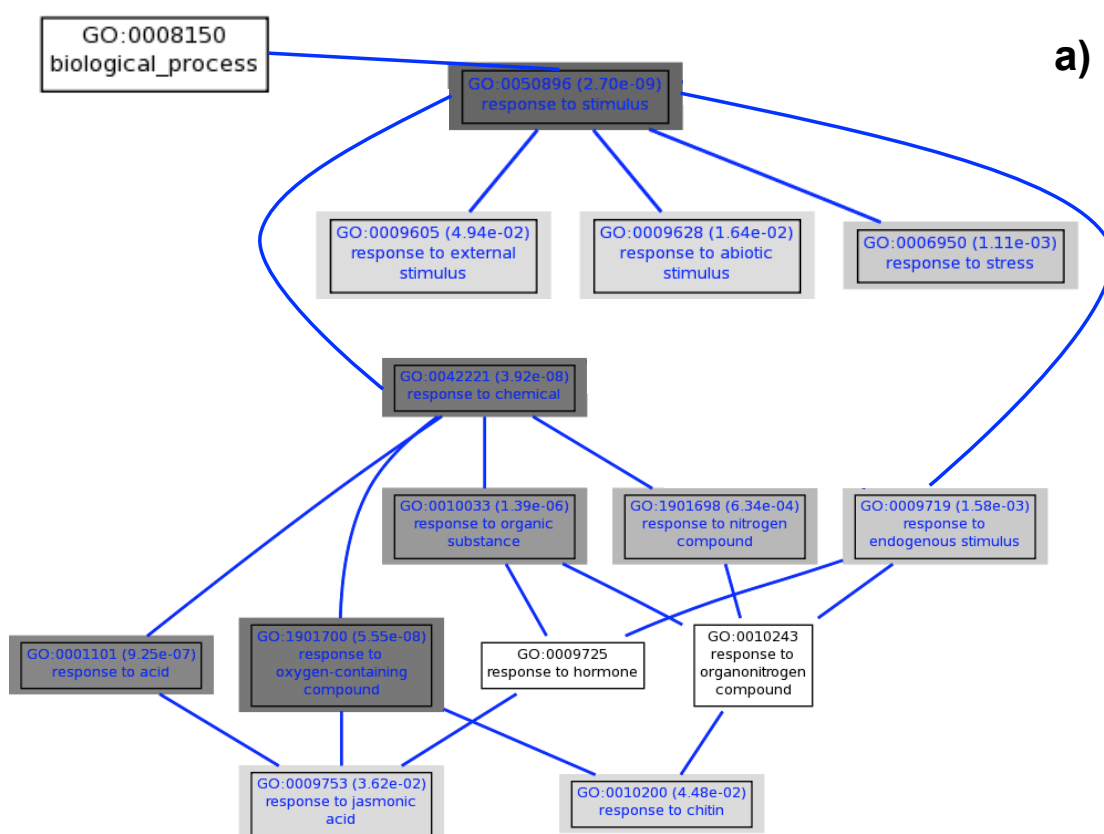


Figure 5.11 DESeq2 and edgeR venn diagram of significantly differentially expressed genes (DEGs), from the interaction (both) between morning and evening and different days (D1-3).

5.3.2.3 Differentially expressed genes (DEGs) in the plant root transcriptome

There was an overlap of 233 DEGs (**Figure 5.11**), from the interaction between morning, evening, and days, between DESeq2 and edgeR analyses (**Appendix Table 5.1**). A number of biological processes were significantly enriched

amongst the DEGs in the evening ($P < 0.05$), including pathways involved with response to jasmonic acid, chitin, abiotic stress, external stress, stimulus and nitrogen, and cell wall organization and biogenesis, single organism cellular process, biological regulation, cation transport, and secondary metabolic processes (**Figure 5.12a-b**). The locations where upregulated genes were expressed, were in the plasma membrane, and extracellular regions (**Figure 5.12c**).



(Full legend on following page)

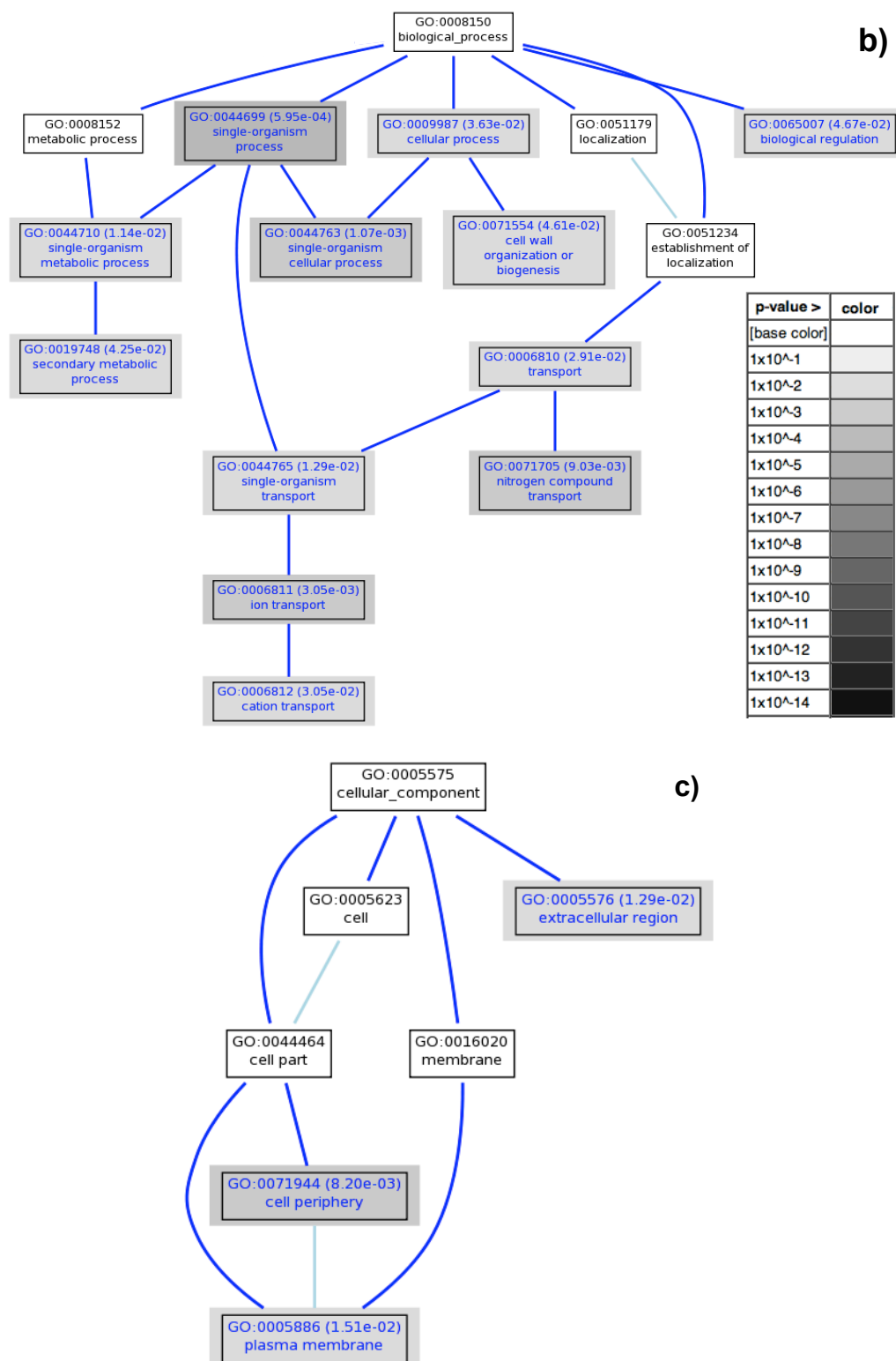


Figure 5.12 a - c. Significantly enriched Gene Ontologies (GOs), annotated with Arabidopsis gene identities, from the interaction between morning and evening and days, generated by the online tool AmiGO, whereby the darker the grey box, stronger the significance (p -value) of that GO enrichment above background levels (see colour key). **Figure 5.12 a** and **b** are the biological processes of the significantly enriched, which were divided in two for presentation purposes. **Figure 5.12c** are the cellular components which the significantly enriched DEGs were found in.

There were three genes, which were significantly differentially expressed between morning and evening in all three days. The genes were a terpenoid cyclase, a high affinity K⁺ transporter, and a cytochrome P450 gene (**Table 5.1**). **Figure 5.13** shows a Venn diagram of the 233 DEGs found in the interaction between mornings and evenings, the three DEGs which are found between every morning and evening, and the circadian clock genes found in the meta-transcriptomes, and the overlaps between these groups, including one circadian clock gene LHY/CCA1 significantly differentially expressed between morning and evening.

Table 5.1 Differentially expressed genes between every morning and evening

Gene Hit	Definition
AT4G02780.1	Terpenoid cyclases/Protein prenyltransferases superfamily protein
AT4G13420.1	High affinity K ⁺ transporter 5
AT1G64940.1	Cytochrome P450, family 87, subfamily A, polypeptide 6

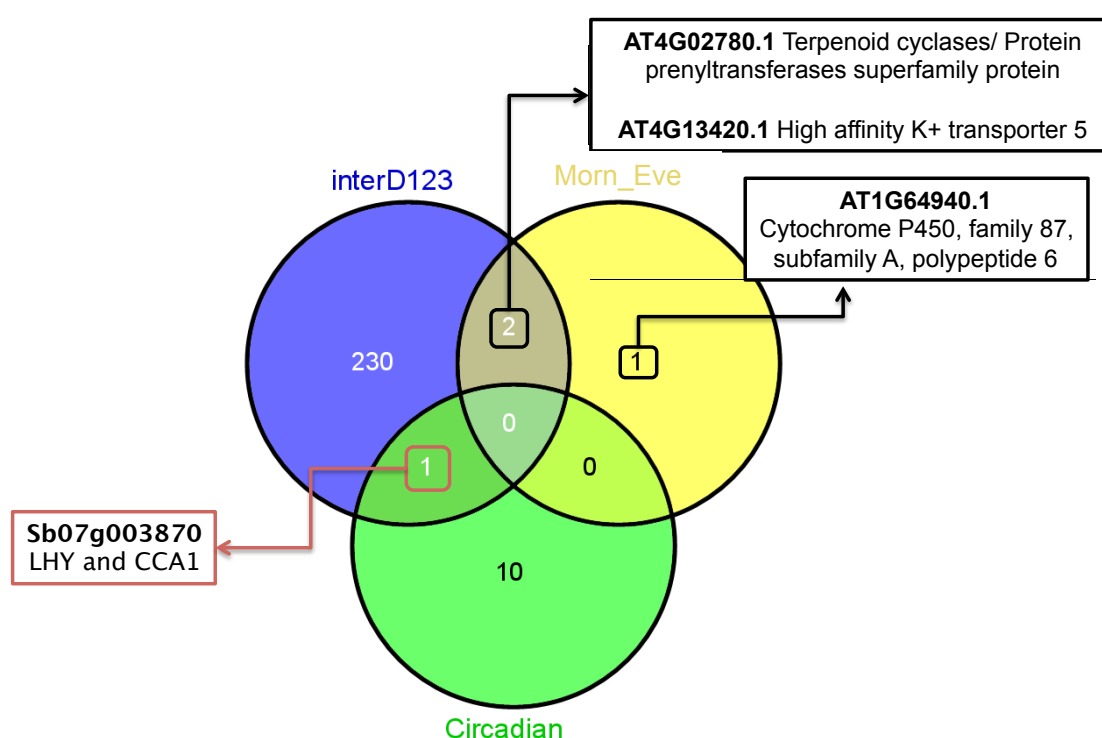
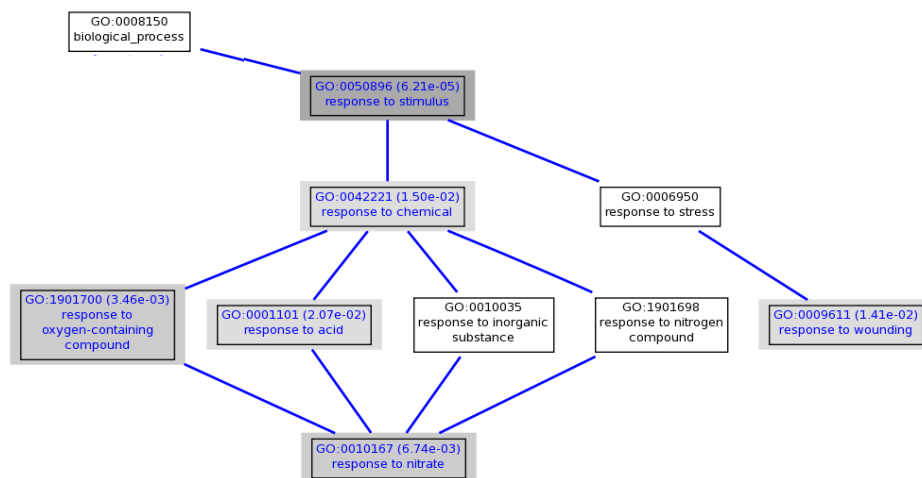
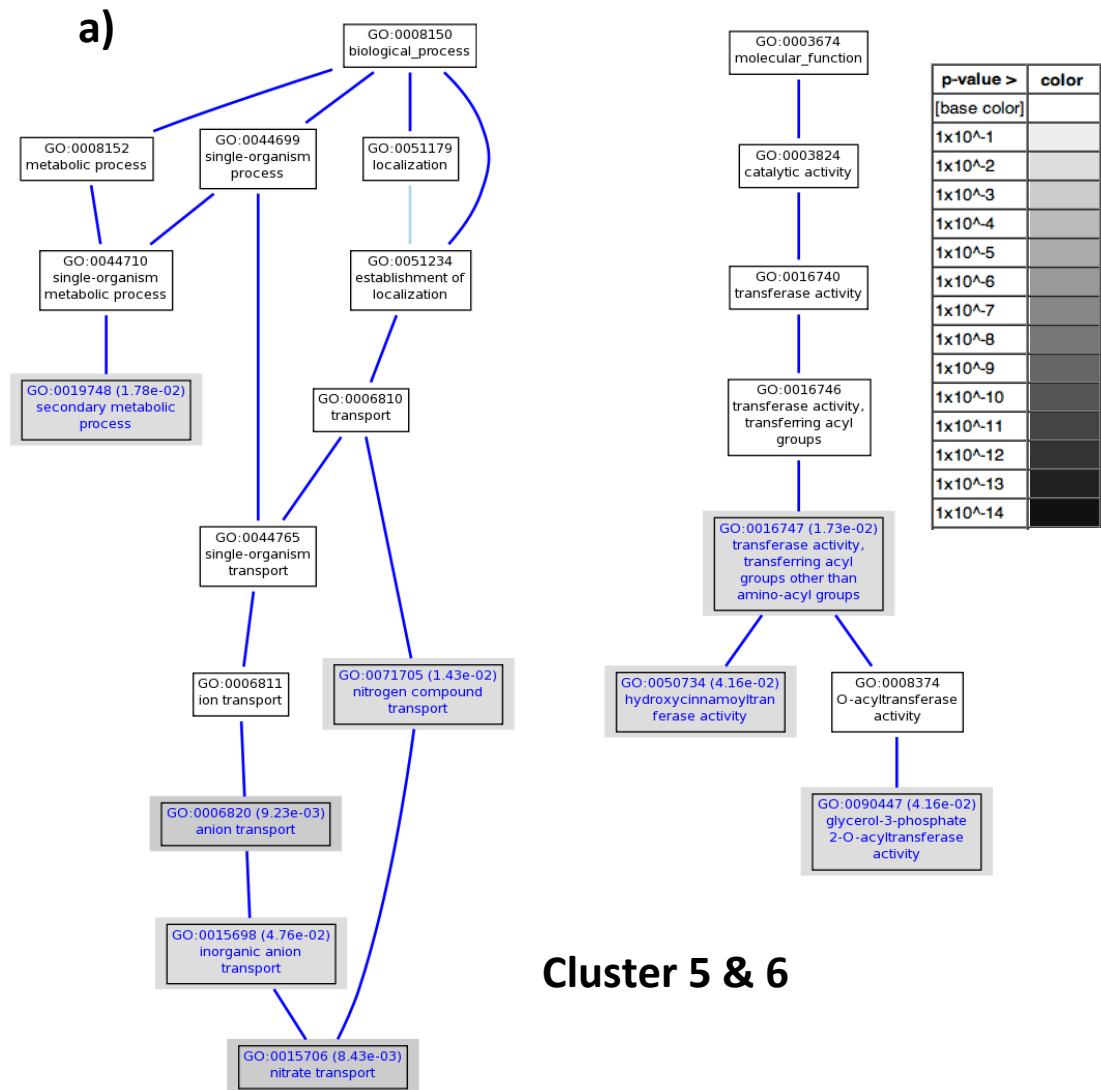


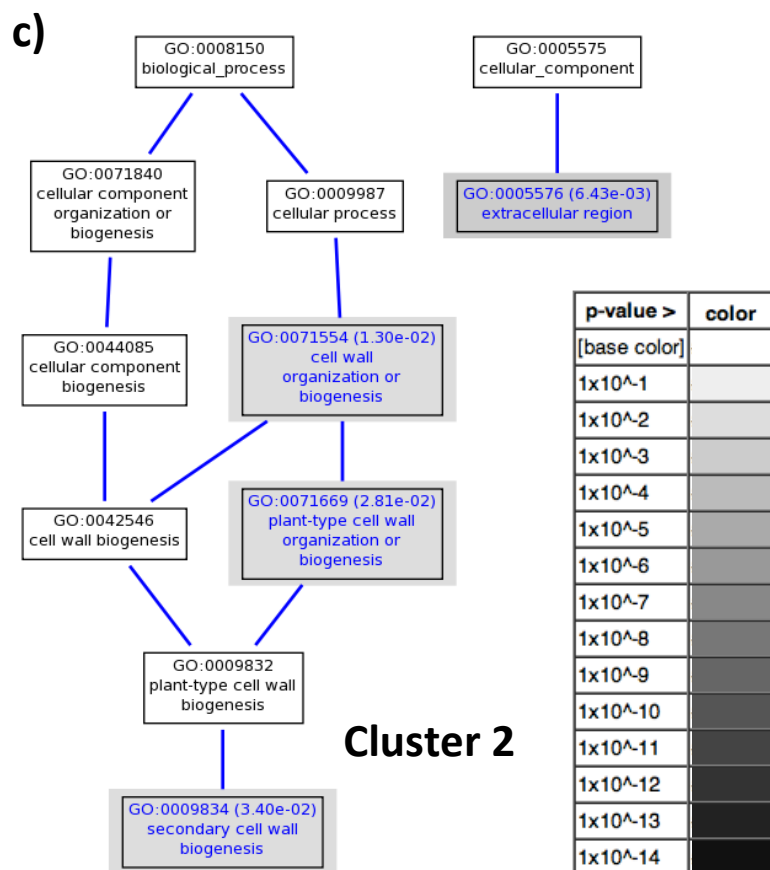
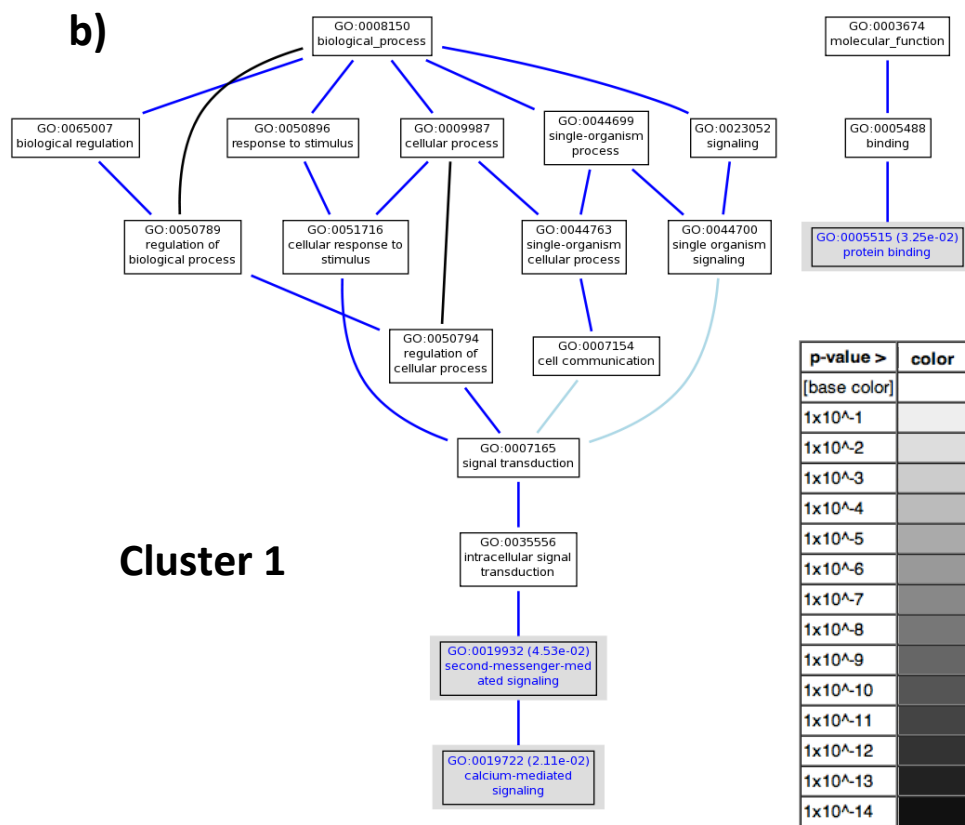
Figure 5.13 Venn diagram of the DEGs from the interaction between morning and evenings and days (interD123), the three DEGs between all three mornings and evenings (Morn_Eve), and the eleven circadian clock gene homologues identified from the *S. bicolor* genome. *S. bicolor* gene identifiers and annotations are provided for the genes in boxes.

5.3.2.4 Chronological DEG gene expression and functional analysis

The gene expression profiles of DEGs were highly variable in expression levels, and oscillated between time points (**Appendix Figure 5.2**). Therefore, expression levels were scaled using Z-scores, and clustered into six groups (**Appendix Figure 5.3**). Clusters 5 and 6 were very similar, and furthermore treated as one (5&6).

Cluster 5&6 had the clearest diurnal expression patterns, with genes more active in the evenings than mornings. There was particularly low expression on the second morning, and high expression on the third evening (**Appendix Figure 5.4a**). There were 84 genes in cluster 5&6, and the most significantly enriched functions in the evenings included nitrate transport, response to nitrate, response to wounding, secondary metabolic processes, and acyl transferase activity (**Figure 5.14a**). Cluster 1 contained 35 DEGs, which were variable in expression pattern across time points, but had very low expression on the final evening (**Appendix Figure 5.4b**). The significantly enriched functions in cluster 1 included calcium-mediated signaling, and protein binding (**Figure 5.14b**). Cluster 2 contained 47 DEGs, which were variable in expression across days, with had fairly average expression in the mornings, but very high expression on the first and final evening, but very low expression on the second evening (**Appendix Figure 5.4c**). The significantly enriched functions in cluster 2 included secondary wall biogenesis, in the extracellular region (**Figure 5.14c**). Cluster 3 contained 40 DEGs, which had low expression on the first day and second morning, and then high expression on the second evening and third day (**Appendix Figure 5.4d**). The significantly enriched functions in cluster 3 included monocarboxylic acid biosynthetic processes and terpene synthase activity (**Figure 5.14d**). Cluster 4 contained 28 DEGs. There was a variety of expression patterns across time in cluster 4, but many genes were clearly oscillating diurnally, either higher in the morning or evening, and all genes were very high on the third evening (**Appendix Figure 5.4d**). There were no significantly enriched functions, however all DEGs in cluster 4, and the other clusters, are in **Appendix Table 5.1**.





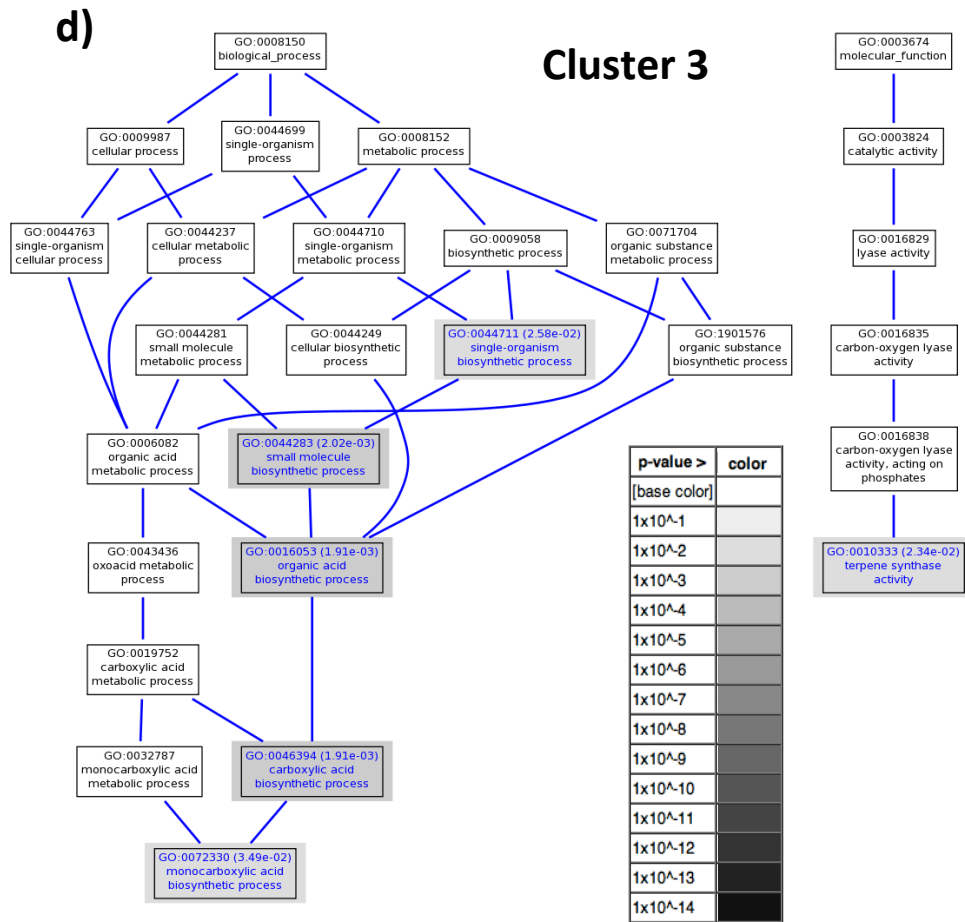
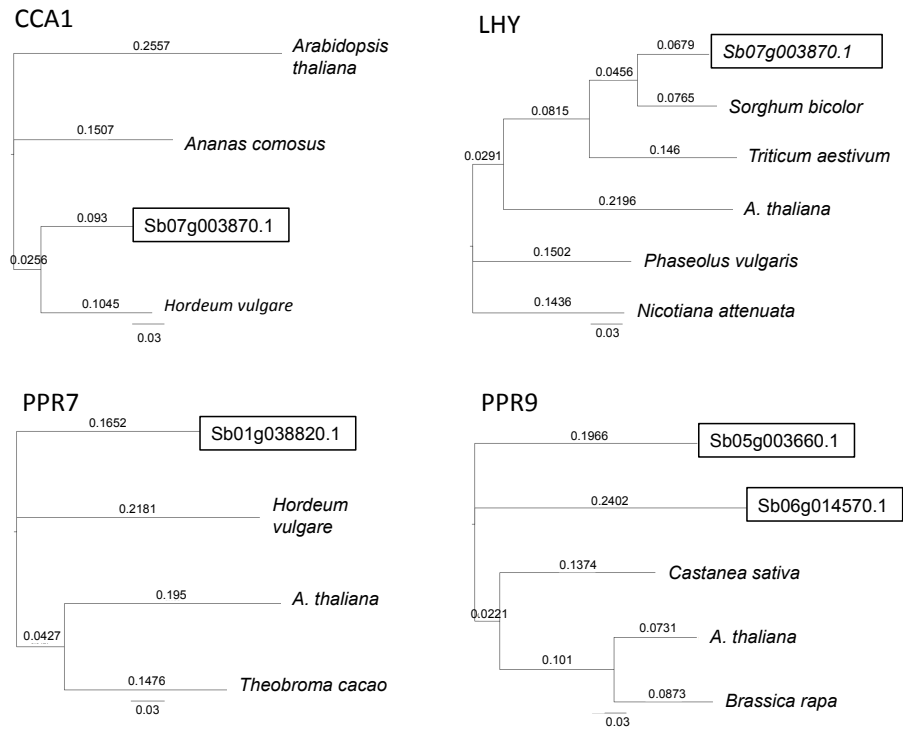


Figure 5.14 a - d. GO term enrichment flow charts, for each of the five clusters, **a)** cluster 5 & 6, **b)** cluster 1, **c)** cluster 2, and **d)** cluster 3. Flow charts are of significantly enriched genes within DEGs ($p < 0.05$), the darker grey the boxes are, the more significant the molecular functions, molecular function, or cellular components are (see key). Boxes at the top of the flow charts are of broader functions, which divide into more specific functions of genes in that group. Boxes contain GO terms. Cluster four contained no significantly enriched functions.

5.3.2.5 Circadian clock gene homologues

Nine *S. bicolor* genes were similar to circadian clock genes (closest plant species, percentage similarity, and genetic distance), which were as follows: Sb07g003870.1 (*H. vulgare* CCA1, 78.9 %, 0.1975), Sb07g003870.1 (*S. bicolor* partial LHY, 100 %, 0.1444) Sb01g038820.1 (*H. vulgare* PRR7, 68 %, 0.3833), Sb05g003660.1 (*C. sativa* PRR9, 79.1 %, 0.3561), Sb03g025560.1 (*A. thaliana* ELF3, 77.1 %, 0.4267), Sb09g030700.1 (*A. thaliana* ELF3, 78.4 %, 0.993), Sb03g003650 (*A. thaliana* GI, 65.2 %, 0.3158), Sb03g047330.1 (*H. vulgare* LUX, 75.0 %, 0.218), Sb03g039610.1 (*A. thaliana* LUX, 74. %, 0.443), and Sb04g026190.1 (*H. vulgare* TOC1, 77.2 %, 0.3599) (**Figure 5.15**).

Morning elements



Evening elements

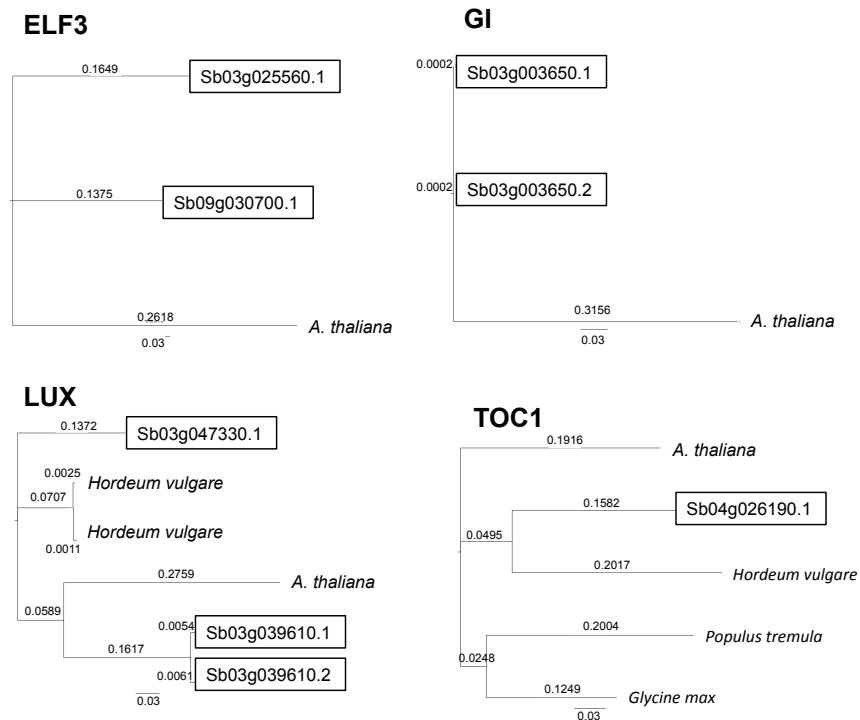


Figure 5.15 Neighbour joining phylogenetic tree of circadian clock genes from different plants species, morning components *CCA1*, *LHY*, *PPR7*, *PPR9*, and evening components *ELF3*, *GI*, *LUX* and *TOC1*, aligned to the closest related *S. bicolor* gene homologues (in boxes). The values on branches and scale bars are of genetic distance

The gene expression heatmap (**Appendix Figure 5.5**), and the normalised heatmap (**Figure 5.16**), show strong variation over time in these circadian clock gene homologues. The homologue matching to both *CCA1* and *LHY* (Sb07g003870.1) was the only circadian homologue, significantly ($P < 0.05$) oscillating between morning and evening, with stronger expression in the morning (**Figure 5.16 and 5.13**). The two *LUX* homologues, *TOC-1*, and *PRR9* & *PRR7*, appeared to be stronger in the evenings than the mornings, however they was no significantly difference (**Appendix Figure 5.5**).

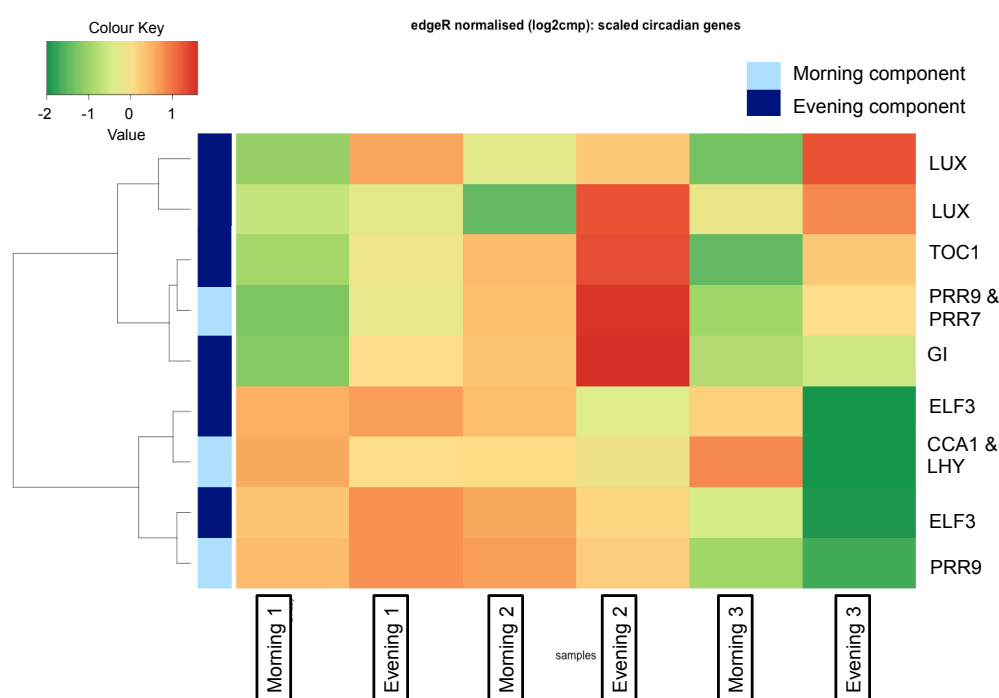


Figure 5.16 Heat map of circadian clock homologues normalised gene expression, using Z-scores, in the *M. giganteus* transcriptome, over three consecutive days. Replicates merged from each morning and evening. Expression profiles were ordered by hierarchical clustering. For gene identities see results section **Circadian clock gene homologues**.

5.3.2.6 Influence of environmental variables on *M. giganteus* gene expression

A number of gene clusters significantly ($P < 0.05$) correlated with environmental variables, particularly with VPD, heat flux, NEE, GPP, and wind direction (see **Figure 5.17** for P -values). Measured VPD had a significant positive correlation with five gene clusters ($P < 0.05$), and significant negative correlation with three clusters. Gap filled sensible heat flux had significant positive correlations with

three clusters, and negative relationships with three ($P < 0.05$). Gap filled NEE had significant negative correlations with five clusters. Total ecosystem respiration had significant negative correlations with five clusters, and positive correlations with five ($P < 0.05$). Gross primary production (GPP) had strong positive relations with four clusters ($P < 0.05$). Corrected soil temperature had four significant negative correlations, and one positive. Soil temperature had three positive, and two negative, significant correlations ($P < 0.05$). Net radiation had one positive, and one negative significant correlation. Wind speed had three significant negative correlations, and wind direction had two significant positive correlations ($P < 0.05$).

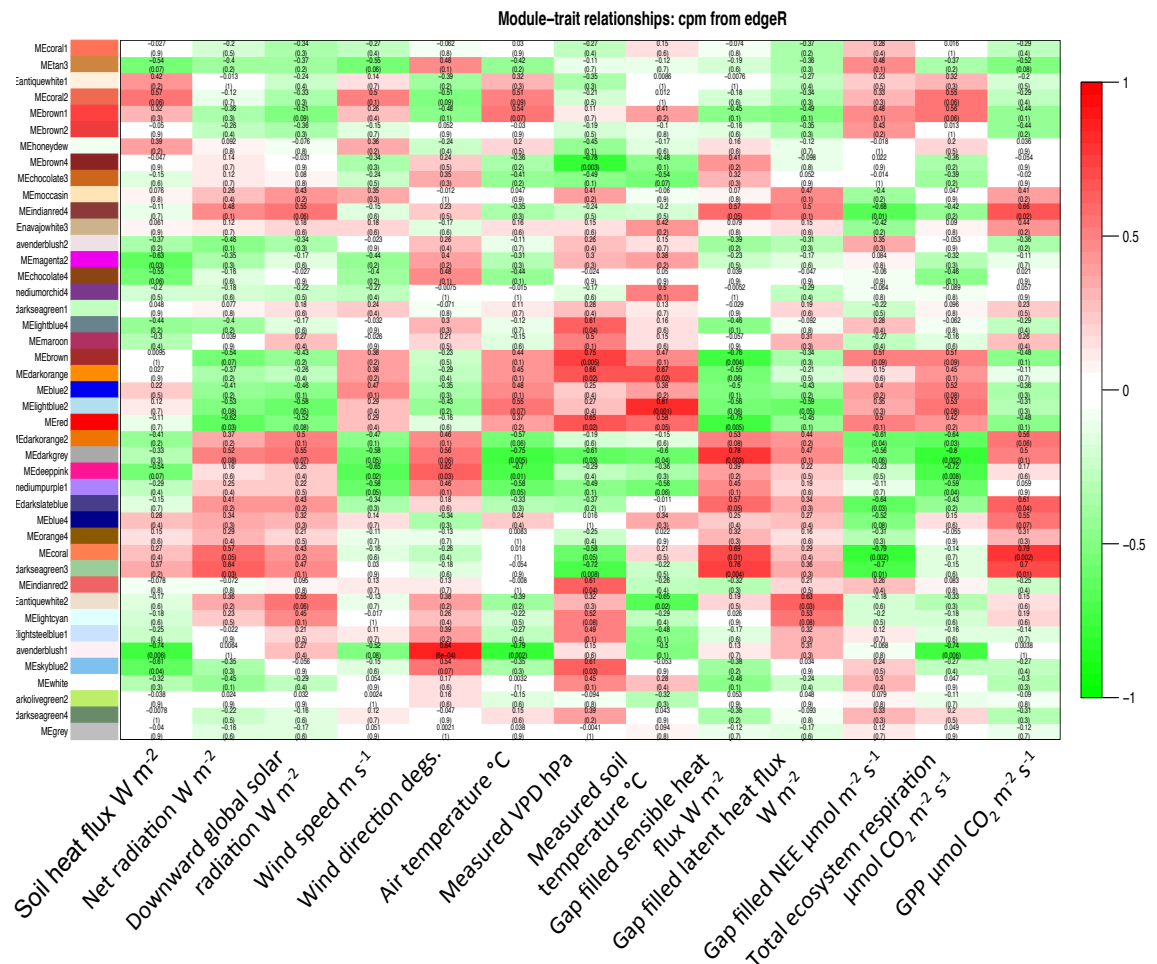


Figure 5.17 Weighted Correlation Gene Network Analysis (WCGNA) of clustered gene expression (Y-axis), with environmental variables (X-axis), negative (deep green) to positive (deep red) Pearson correlation (see scale). Correlation coefficients, and significance values (P) of the correlation, are written inside each box for each relationship.

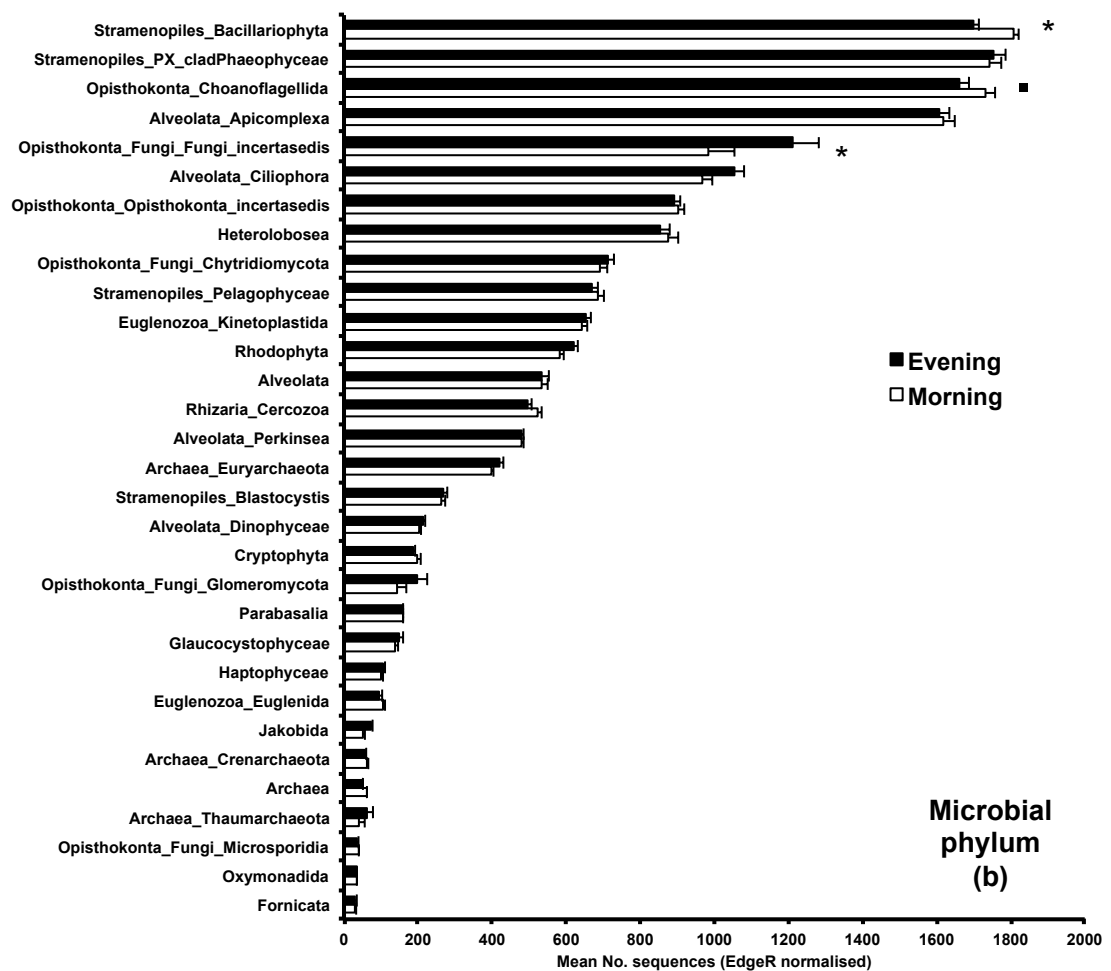
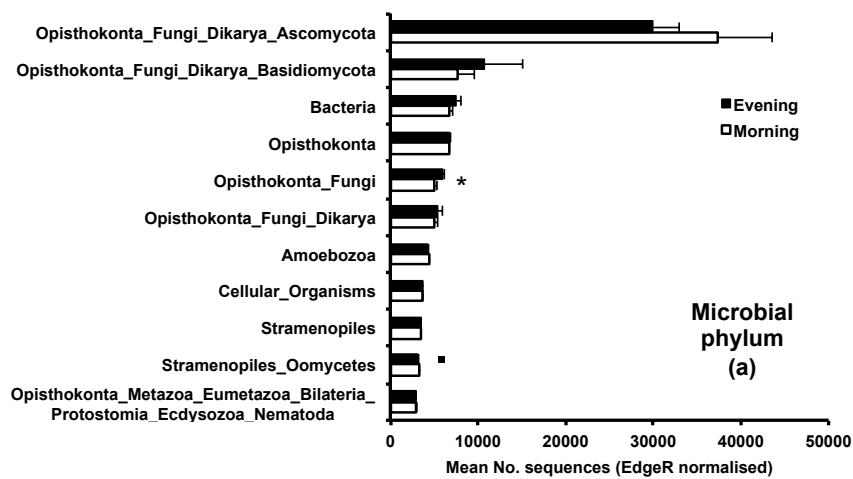
5.3.3 Meta-transcriptome analysis of root microbes

A total of 2,755,263 edgeR normalised mRNA reads passed quality filtering and were successfully aligned to the microbial database, including fungi, bacteria, archaea, and other eukaryotes. The number of reads per sample ranged between 131,819, and 387,187, with a mean of 229,605. After normalisation with edgeR, there was a total of 2,627,972 reads, which ranged from 200,439 to 251,940 per sample, and a mean of 218,998.

5.3.3.1 Meta-transcriptome microbial taxonomy

Altogether, there were 89 phyla, 156 classes, 366 orders, 606 families, 933 genera, and 1,397 species, in the microbial meta-transcriptome. The phyla with the most active transcription in morning (M) and evening (E) (mean no. seqs \pm SEM) were the Ascomycota (M = 37,292 \pm 6,265, E = 29,958 \pm 2,993) Basidiomycota (M = 7,642 \pm 2,017, E = 10,686 \pm 4,440), Bacteria (M = 6,713 \pm 394, E = 7,564 \pm 584), Opisthokonta (M = 6,698 \pm 99, E = 6,720 \pm 146), fungi (M = 5,013 \pm 309, E = 5,952 \pm 212), Dikarya fungi (M = 5,050 \pm 378, E = 5,464 \pm 475), Amoebozoa (M = 4,405 \pm 51.5, E = 4,268 \pm 87.5), Stramenopiles (M = 3,545 \pm 38.3, E = 3,475 \pm 85.2), Oomycetes (M = 3,319 \pm 52.0, E = 3,156 \pm 73.0), and Nematoda (M = 2,956 \pm 65.5, E = 2,862 \pm 112.3) (**Figures 5.18**). The most dominant microbial group was 'Eukaryota' (mean no. seqs \pm SEM, M = 118,620 \pm 943, E = 115,165 \pm 1460), which was a mixture of conserved eukaryote genes.

A number of microbial phyla had significantly (TTEST, * = $P < 0.05$) more activity in the evening, including chordate ($P = 0.013$ *), fungi ($P = 0.031$ *), fungi incertae sedis ($P = 0.044$ *), and to 10 % significance (. = $P < 0.1$), malawimonadidae ($P = 0.072$.), whereas other phyla were more active in the morning, including, bacillariophyta ($P = 0.034$ *), and fungi environmental sample ($P = 0.022$ *), and others to 10 %: oomycetes ($P = 0.099$.), and choanoflagellida ($P = 0.099$.). The results which are below 10 % significance level are highlighted as the sample size was small, and it indicates a small shift, which may be of interest in this novel work.



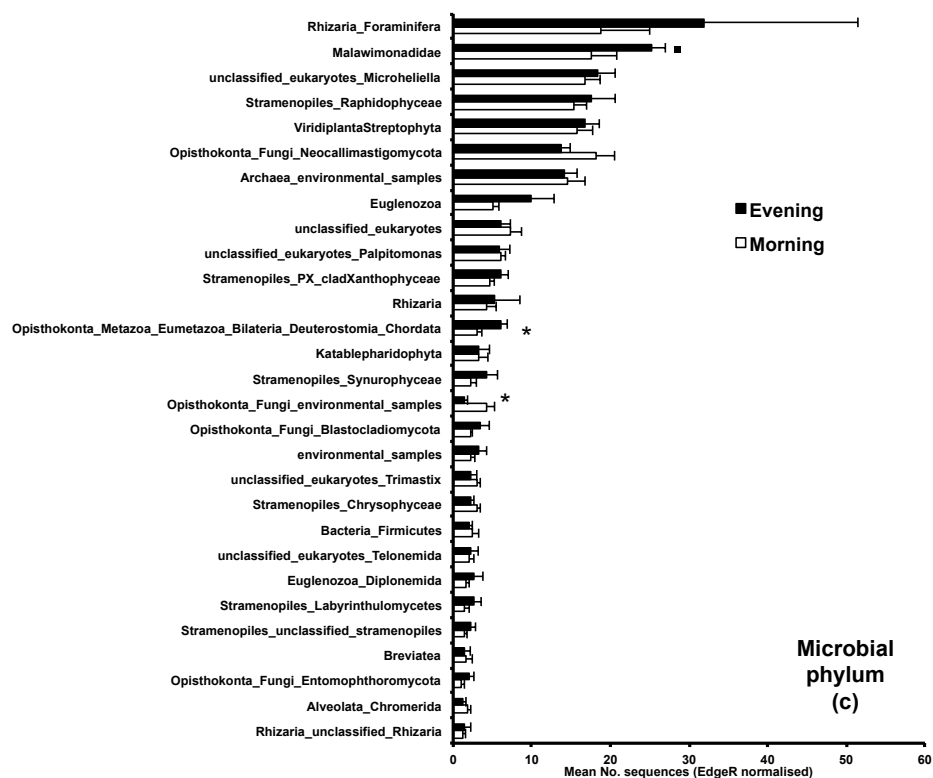


Figure 5.18 EdgeR normalised read counts of eukaryotic, bacterial, and archeal, phyla in the meta-transcriptome, of phyla with a mean of **a)** over 2,000 reads, **b)** 31 – 2,000 reads, and **3)** 1 – 30 reads. Where, error bars are SEM, and TTEST significance values are represented by ■ ($P < 0.1$), * ($P < 0.05$), for the difference between morning and evening means.

5.3.3.2 Meta-transcriptome analysis of fungal taxonomy

A total of 2,757,814 subsampled mRNA reads were successfully aligned to the fungal database. The number of reads per sample ranged between 189,704 and 267,878, with a mean of 229,817. Altogether, there were 13 phyla, 50 class, 133 orders, 256 families, 436 genus, and 652 species. The largest groups were conserved eukaryote genes, and ‘fungi’ representing conserved fungal genes, which represented a mean (\pm SEM) of 55.4 % (\pm 0.53) and 21.1 % (\pm 1.3) of reads respectively. Large numbers of reads may have been classified at conserved levels because the classification method was Lowest Common Ancestor, which assigns taxonomy to the lowest common ancestor of the whole data set (Huson, *et al.*, 2007), and therefore many common gene functions may have been assigned to a higher taxonomy level. From the remaining identified mRNA, the phyla with the most active transcription in morning (M) and evening (E) (mean relative abundance % \pm SEM, TTEST) were the Ascomycota (M = 52.2 ± 2.4 , E = 47.0 ± 1.9 , $P = 0.176$), Dikarya (M = 25.2 ± 0.9 , E = 26.7 ± 1.0 , P

= 0.715), Basidiomycota ($M = 15.3 \pm 1.8$, $E = 18.2 \pm 2.3$, $P = 0.873$), Fungi incertae sedis ($M = 3.3 \pm 0.2$, $E = 3.8 \pm 0.2$, $P = 0.425$), Chytridiomycota ($M = 3.3 \pm 1.5$, $E = 3.9 \pm 0.2$, $P = 0.255$), and Glomeromycota ($M = 0.28 \pm 0.02$, $E = 0.42 \pm 0.03$, $P = 0.491$) (**Appendix Figure 5.6a.**). The dominant fungal classes, were the dikarya divisions Sordariomycetes ($M = 7.6 \pm 0.4$, $E = 10.6 \pm 0.4$, $P = 0.085$), Agariomycetes ($M = 4.4 \pm 2.4$, $E = 10.8 \pm 2.1$, $P = 0.636$), Leotiomyceta ($M = 11.6 \pm 0.58$, $E = 7.7 \pm 0.48$, $P < 0.351$), Dothideomycetes ($M = 16.3 \pm 2.5$, $E = 4.3 \pm 0.8$, $P = 0.086$), and Saccharomycetes ($M = 3.7 \pm 0.2$, $E = 4.5 \pm 0.2$, $P = 0.733$) (**Appendix Figure 5.6b.**). The dominant active fungal species, were most related to a Pleosporineae sp. ($M = 8.4 \pm 1.1$, $E = 2.5 \pm 0.3$, $P = 0.083$), *Metarhizium anisopliae* ($M = 3.4 \pm 0.2$, $E = 4.2 \pm 0.2$, $P = 0.149$), *Batrachochytrium dendrobatidis* ($M = 3.2 \pm 0.1$, $E = 3.4 \pm 0.2$, $P = 0.727$), *Rhizopus delemar* ($M = 2.9 \pm 0.1$, $E = 3.6 \pm 0.1$, $P = 0.217$), *Parastagonospora nodorum* ($M = 2.6 \pm 0.3$, $E = 0.76 \pm 0.2$, $P = 0.079$), and *Coniophora puteana* ($M = 0.08 \pm 0.732$, $E = 1.1 \pm 0.008$) and *Moniliophthora perniciosa* ($M = 0.49 \pm 0.03$, $E = 0.63 \pm 0.04$, $P = 0.146$) (**Appendix Figure 5.6c.**).

5.3.3.3 Fungal community similarity in the morning and evening

At more refined levels of hierarchy, there was a lower level of taxonomic resolution. However, the community structure of the four highest levels of fungal taxonomy (species, genus, family, order) were significantly different ($P < 0.1$), between morning and evening, when morning and evening profiles were compared using ADONIS (Bray Curtis dissimilarity), including order ($r^2 = 0.182$, $P = 0.090$.), family ($r^2 = 0.183$, $P = 0.084$.), genus ($r^2 = 0.181$, $P = 0.078$.), and species ($r^2 = 0.180$, $P = 0.090$.) (**Table 5.2**). Fungal community structures at phyla ($r^2 = 0.195$, $P = 0.108$), and class ($r^2 = 0.179$, $P = 0.114$) hierarchies, were not significantly different from morning to evening (**Table 5.2**). However, **Figure 5.19** shows that morning and evening activity of fungal communities shifted at all taxonomic levels. The top ten orders in the SIMPER analysis, contributed 80.6 % of total dissimilarity, between morning and evening. A number of orders were higher in the morning (proportion higher than evening), which included Pleosporales (328.9 %), Boletales (415.0 %), and Helotiales (23 %), whereas the Agaricomycetes and Agaricales were 65.5 % and 39.5 % higher in the evening, respectively (**Table 5.3**). The top ten dissimilar families contributed 73.2 % to total dissimilarity, and included Pleosporinae and Phaeosphaeriaceae (both 3513 %), and Coniophoraceae (1209.6 %). The top ten dissimilar genera

contributed 72.4 % to dissimilarity, and included *Parastagnospora* (351.6 %), and *Coniphora* (1209.6 %) (**Table 5.3**). The top ten dissimilar species contributed 71.6 % to dissimilarity, and included *Parastagnospora nodorum* (351.5 %), and *Coniphora puteana* (1209.6 %) (**Table 5.3**).

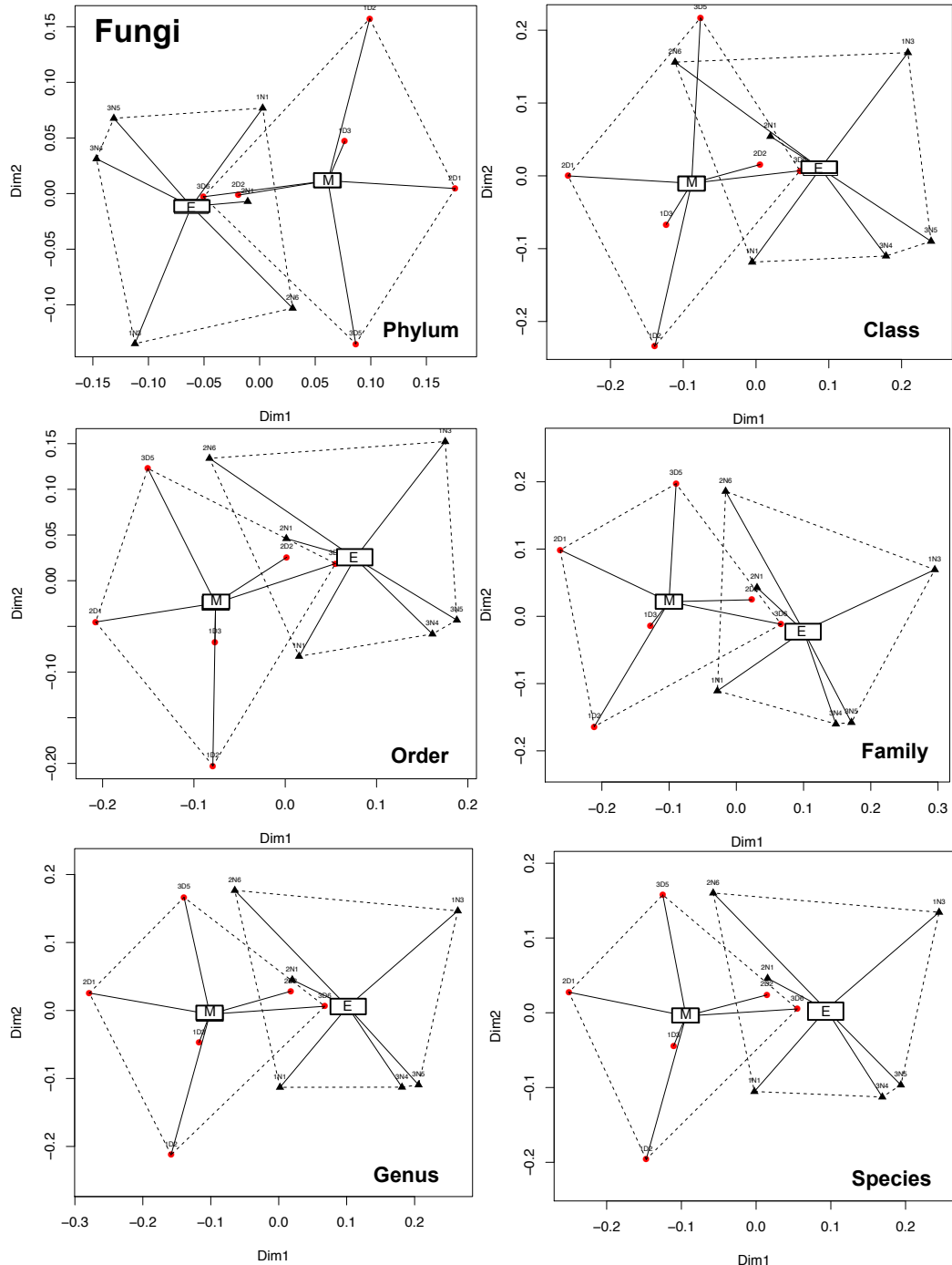


Figure 5.19 NMDS plot of fungal mRNA expression during the morning (M) and evening (E), where functional genes were assigned to phyla, class, order, family, genus, and species level. Points represent community profiles of each sample, where red circles are morning samples, and black triangles are evening samples. In data point labels, the first number represents the sampling day (1-3), and the final number is the replicate number.

Table 5.2. Summary statistics of the differences between morning and evening fungal gene expressions assigned to varying levels of taxonomic classification, using ADONIS (Bray-Curtis dissimilarity), where . represents $P < 0.1$ which means taxonomic profiles are significantly different between morning and evening.

Division	ADONIS (Bray Curtis)	
	r^2	P
Phylum	0.195	0.108
Class	0.179	0.114
Order	0.182	0.090 .
Family	0.183	0.084 .
Genus	0.181	0.078 .
Species	0.180	0.090 .

Table 5.3. Top ten taxa contributing to differences in morning and evening fungal mRNA transcription, using SIMPER analysis, separated into the following divisions: order, family, genus, and species, where Av Morn and Av Eve represent the average read counts in the morning and evening respectively, and Cum Sum is the cumulative sum of the contribution each taxonomic group adds to dissimilarity between morning and evening. Significance values between morning and evening in previous table (5.2).

Fungal Order	Av Morn	Av Eve	Cum Sum	Fungal Family	Av Morn	Av Eve	Cum Sum
Fungi	51308	45960	0.284	Fungi	51308	45960	0.277
Pleosporales	6260	1904	0.467	Pleosporineae	4285	1250	0.402
Leotiomyceta	5635	4140	0.544	Leotiomyceta	5635	4140	0.478
Dikarya	14339	13383	0.611	Dikarya	14339	13383	0.544
Agaricomycetes	1366	2255	0.676	Agaricomycetes	1366	2255	0.606
Agaricales	891	1243	0.706	Phaeosphaeriaceae	1326	378	0.645
Pezizomycotina	2573	1966	0.735	Pezizomycotina	2573	1966	0.674
Boletales	893	215	0.763	Coniophoraceae	696	58	0.698
Helotiales	889	720	0.787	Sordariomycetes	956	1255	0.716
Sordariomycetes	956	1255	0.806	Basidiomycota	1919	1977	0.732

Fungal Genus	Av Morn	Av Eve	Cum Sum	Fungal Species	Av Morn	Av Eve	Cum Sum
Fungi	51308	45960	0.275	Fungi	51308	45960	0.272
Pleosporineae	4285	1250	0.398	Pleosporineae	4285	1250	0.393
Leotiomyceta	5635	4140	0.473	Leotiomyceta	5635	4140	0.468
Dikarya	14339	13383	0.538	Dikarya	14339	13383	0.532
Agaricomycetes	1366	2255	0.600	Agaricomycetes	1366	2255	0.594
Parastagonospora	1325	377	0.639	Parastagonospora nodorum	1325	377	0.632
Pezizomycotina	2573	1966	0.667	Pezizomycotina	2573	1966	0.660
Coniophora	696	58	0.691	Coniophora puteana	696	58	0.683
Sordariomycetes	956	1255	0.708	Sordariomycetes	956	1255	0.701
Basidiomycota	1919	1977	0.724	Basidiomycota	1919	1977	0.716

5.3.3.4 Fungal functional gene expression analysis

A total of 25,369 subsampled fungal sequences were functionally annotated from the meta-transcriptome. A number of functions were annotated with SEED; 19 at Level 1, 30 at Level 2, 93 at Level 3, and 131 at Level 4 (**Appendix Figure 5.7a-d**). At SEED Level 1 (**Appendix Figure 5.7a**), the most dominant (proportion of reads) fungal functions involved protein metabolism (50.3 %),

RNA metabolism (23.0 %), stress response (7.1 %), nitrogen metabolism (3.4 %), cell wall and capsule (2.3 %), metabolism of aromatic compounds (2.1 %), amino acids and derivatives (2.1 %), fatty acids, lipids, and isoprenoids (2.0 %), DNA metabolism (1.7 %), respiration (1.4 %), carbohydrates (1.2 %), and secondary metabolism (1.1 %).

At SEED Level 2 (**Appendix Figure 5.7b**), the most dominant (proportion of reads) fungal functions involved protein biosynthesis (44.9 %), transcription (16.7 %), RNA processing and modification (5.6 %), heat shock dnaK (3.3 %), protein folding (3.3 %), nitrosative stress (2.6 %), phospholipids (1.9 %), urea cycle polyamines (1.6 %), ammonia assimilation (0.7 %), and aminosugars (0.4 %) (**Appendix Figure 5.7b**).

At SEED Level 3 (**Appendix Figure 5.7c**), the most dominant (proportion of reads) functions included translation initiation factors (18.9 %), RNA polymerase initiation factors (11.1 %), translation elongation factors (7.7 %), Universal GTPases (6.9 %), ribosome biogenesis (3.4 %), protein chaperones (2.2 %), flavohemoprotein (1.7 %), mycolic acid synthesis (1.5 %), chaperone protein DnaJ (1.4 %), and urea decomposition (1.3 %) (**Appendix Figure 5.7c**).

Finally, at SEED Level 4 the most dominant (proportion of reads) functions included TATA-box binding protein (11.0 %), Eukaryotic translation initiation factors 5A (8.2 %), 2 (3.8 %), 6 (3.0 %), and G (2.8 %), fibrillarin (3.4 %), flavohemoprotein (1.7 %), chaperone proteins DnaJ (1.4 %) and DnaK (0.7 %), urease accessory protein UreG (1.2 %), acyl carrier protein (1.2 %), and glutaredoxin (0.7 %) (**Appendix Figure 5.7d**).

5.3.3.5 Fungal functional expression changes in the morning and evening

Fungal functional expression was significantly different, at $P < 0.05$ using ADONIS (Bray Curtis dissimilarity), between morning and evening at all SEED Levels (L1 – L4): L1 ($r^2 = 0.259$, $P = 0.034$ *), L2 ($r^2 = 0.231$, $P = 0.025$ *), L3 ($r^2 = 0.227$, $P = 0.23$ *), ($r^2 = 0.207$, $P = 0.028$ *) (**Table 5.4**). The shift between morning and evening communities is clear at all levels in the NMDS plot, as morning and evening profiles cluster separately (**Figure 5.20** and **Table 5.4**).

At SEED L1, the top ten functions contributed to 93.4 % of dissimilarity, and most functions were higher in the morning (proportion higher than evening), including protein metabolism (13.4 %), stress response (92.9 %), RNA metabolism (13.2 %), nitrogen metabolism (308.6 %) metabolism of aromatic compounds (94.9%), cell wall and capsule (66.4 %), carbohydrates (91.7 %), fatty acids, lipids and isoprenoids (74.0 %), and amino acids and derivatives (30.1 %), and one function was higher in the evening; respiration which was 34.4 % higher in the evening than the morning (**Table 5.5**).

At SEED L2 the top ten functions contributed to 84.2 % of dissimilarity, and mostly functions were higher in the morning (proportion higher than evening), including protein biosynthesis (11.9 %), transcription (15.7 %), nitrosative stress (548.6 %), catabolism of aromatic compounds (2.1 times more), cell wall of mycobacteria (63.8 %), phospholipids (73.3 %), amino sugars (475.0 %), urea cycle polyamines (31.9 %), and one function was higher in the evening; electron donating reactions by 40.1 % (**Table 5.5**).

At SEED L3 the top ten functions contributed to 78.4 % of dissimilarity between morning and evening (**Table 5.5**). The L3 functions higher in the morning (proportion higher than evening) included translation initiation factors (23.1 %), RNA polymerase III (15.6 %), flavohemoprotein (548.6 %), chaperone protein DnaJ (44.4 %), mycolic acid synthesis (63.8 %), and fatty acid synthesis (73.3 %), whereas the following functions were more active in the evening (proportion higher than morning): translation elongation factors (9.6 % and 15.5 %), Universal GTPases (15.2 %), and respiration complex 1 (46.6 %) (**Table 5.5**).

At SEED L4 the top ten functions contributed to 72.5 % of dissimilarity between morning and evening (**Table 5.5**), which were mostly higher in the morning (proportion higher than evening) including TATA-box binding protein (15.6 %), flavohemoprotein (548.6 %), translation initiation factors 5A (21.7 %), 2 gamma subunit (16.5 %), G (2.5 %), 6 (19.4 %), quinate permease (208.9 %), eukaryote peptide chain release (16.9 %), and acyl carrier protein (73.3 %), and one function, translation elongation factor 2, was 31.1 % higher in the evening.

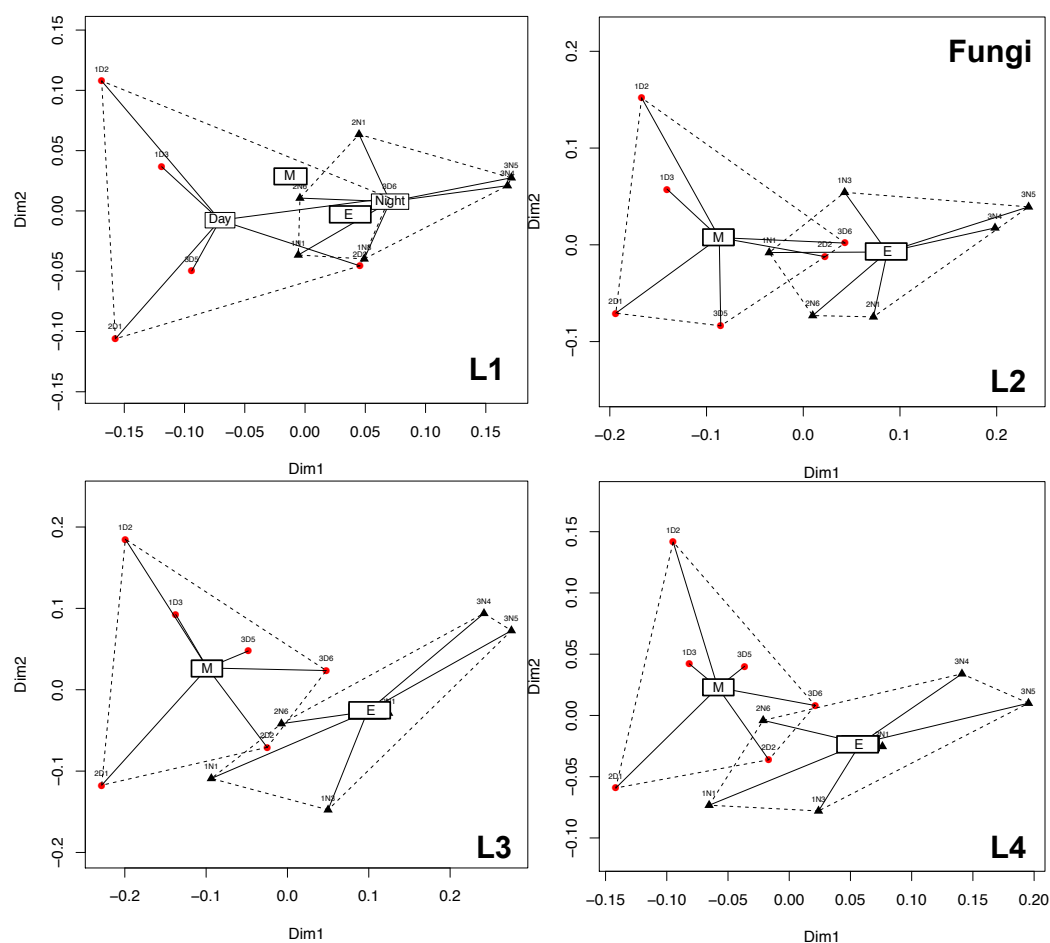


Figure 5.20 NMDS plot of fungal morning (M) and evening (E) expressed genes, with SEED functional annotation, Level (L) 1 – 4, see **Appendix Figure 5.7a-d** for gene identities and abundances. Points represent community profiles of each sample, where red circles are morning samples, and black triangles are evening samples. In data point labels, the first number represents the sampling day (1-3), and the final number is the replicate number.

Table 5.4 ADONIS (Bray Curtis) differences between morning and evening fungal functions, separated into SEED Level (L) 1 – 4, where * represents $P < 0.05$ which means functional profiles are significantly different between morning and evening.

SEED Level	ADONIS (Bray Curtis)	
	r^2	P
L1	0.259	0.034 *
L2	0.231	0.025 *
L3	0.227	0.023 *
L4	0.207	0.028 *

Table 5.5 SIMPER analysis of top contributing discriminating functions between morning and evening group averages, SEED level (L) 1-4, where Av Morn and Av Eve represent the average read counts in the morning and evening respectively, and Cum Sum is the cumulative sum of the contribution each function adds to dissimilarity between morning and evening. Significance values between morning and evening in previous table (5.4).

Fungal SEED L1	Av Morn	Av Eve	Cum Sum	Fungal SEED L2	Av Morn	Av Eve	Cum Sum
Protein Metabolism	1131	997	0.254	Protein biosynthesis	1035	925	0.203
Stress Response	198	103	0.422	Transcription	390	337	0.335
RNA Metabolism	517	456	0.589	Nitrosative stress	96	18	0.539
Nitrogen Metabolism	108	35	0.710	Electron donating reactions	24	33	0.578
Metabolism of				Protein folding	81	62	0.655
Aromatic Compounds	58	30	0.760	Catabolism of Aromatic Compounds	48	23	0.692
Respiration	26	35	0.806	Cell wall of Mycobacteria	60	37	0.722
Cell Wall and Capsule	62	37	0.844	RNA processing and modification	126	119	0.752
Carbohydrates	35	18	0.878	Phospholipids	52	30	0.809
Fatty Acids, Lipids, and Isoprenoids	53	30	0.913	Aminosugars	16	3	0.826
Amino Acids and Derivatives	50	39	0.934	Urea cycle polyamines	47	36	0.842

Fungal SEED L3	Av Morn	Av Eve	Cum Sum	Fungal SEED L4	Av Morn	Av Eve	Cum Sum
Translation initiation factors eukaryotic and archaeal	686	557	0.129	TATA-box binding protein	387	335	0.240
RNA polymerase III initiation factors	387	335	0.402	Flavohemoprotein	96	18	0.366
Flavohemoprotein	96	18	0.473	Eukaryotic translation initiation factor 5A	297	244	0.421
Translation elongation factors eukaryotic and archaeal	242	265	0.579	Translation elongation factor 2	109	142	0.512
Universal GTPases	210	241	0.611	Eukaryotic translation initiation factor 2 gamma subunit	145	124	0.537
Translation elongation factor G family	203	235	0.642	Quinate permease	43	21	0.599
Respiratory Complex I	22	32	0.669	Eukaryotic peptide chain release factor GTP-binding subunit	85	73	0.618
Chaperone protein DnaJ	56	39	0.743	Translation elongation factor G	95	92	0.656
Mycolic acid synthesis	60	37	0.764	Acyl carrier protein	52	30	0.708
Fatty Acid Biosynthesis FASII	52	30	0.784	Eukaryotic translation initiation factor 6	107	90	0.725

5.3.3.6 Fungal transcriptomes

Meta-transcriptome reads were aligned to three genomes, *Stagonospora nodorum*, *Coniphora puteana*, and *Rhizophagus irregularis*. A total of 3,135,768 reads were aligned to *S. nodorum*, with mean of 261,314 (\pm 88,380 SEM). A total of 171,064 reads were aligned to *C. puteana*, with mean of 14,255 (\pm 4508 SEM). A total of 742,761 reads were aligned to *R. irregularis*, with mean of 61,896 (\pm 8,328 SEM). A number of genes were then identified in the aligned transcriptomes, which are described below.

5.3.3.7 Fungal circadian clock homologues in fungal transcriptome alignments

Most of the fungal circadian clock homologues were identified in the transcriptomes of *S. nodorum*, *C. puteana*, and *R. irregularis*, from the root meta-transcriptome. *N. crassa FRH* had homology (percentage identity, genetic

distance) to transcripts from: *S. nodorum* (61.7 %, 0.397), *C. puteana* (50.5 %, 0.512), and *R. irregularis* (43.7 %, 1.044) (**Figure 5.21**). *N. crassa* FWD-1 had homology (percentage identity and genetic distance) to transcripts from *S. nodorum* (54.2 %, 1.497), and *C. puteana* (68.4 %, 0.607), but no homologous reads were found in *R. irregularis* transcripts. *Verticillium alboatrum* VVD had homology (percentage identity and genetic distance) to *S. nodorum* (54.6 %, 1.126), *C. puteana* (47.9 %, 1.305), and *R. irregularis* (60.9 %, 1.480). *Phaeosphaeria* sp. Sn23-1 WC-1 had homology (percentage identity and genetic distance) to *S. nodorum* (98.8 %, 0.003), and *R. irregularis* (45.8 %, 0.847), and *C. puteana* (55.5 %, 0.762) had closest homology (percentage identity and genetic distance) to *Agaricus bisporus*. *Phaeosphaeria* sp. Sn23-1 WC-2 had homology (percentage identity and genetic distance) to transcripts from *S. nodorum* (98.6 %, 0.002), and *R. irregularis* (26.9 %, 1.471), and *C. puteana* (45.3 %, 1.358) and homology to *N. crassa*. *N. crassa* FRQ only had homology (percentage identity and genetic distance) to *S. nodorum* (34.1 %, 3.825), and no homologous transcripts were found from either *C. puteana* or *R. irregularis* alignments (**Figure 5.21**).

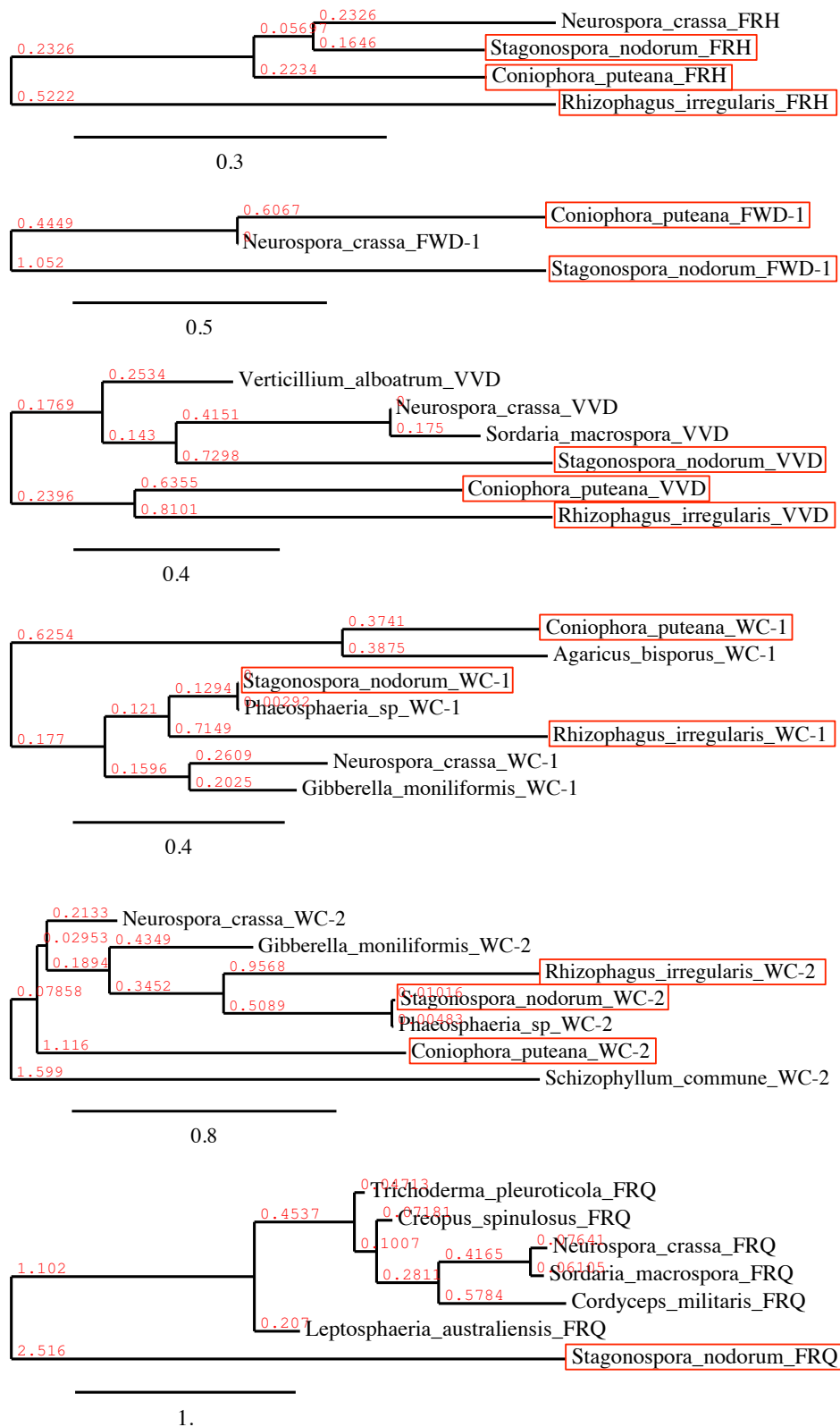


Figure 5.21 Neighbour joining phylogenetic trees of the distance between a number of fungal circadian clock homologues, with the top scoring matches (circadian clock-like genes) from the three fungal transcriptome alignments in red boxes, *Stagonospora nodorum*, *Coniophora puteana*, and *Rhizophagus irregularis*. From the top tree down, the circadian homologues were *FRH*, *FWD-1*, *VVD*, *WC-1*, *WC-2*, and *FRQ*. Values on branches, and scale bars, represent genetic distance.

5.3.3.8 Fungal circadian clock homologues in the meta-transcriptome

The circadian homologues with the highest number of reads in the meta-transcriptome (subsampled mean \pm SEM), were FRH (123 ± 9.0), and WC-1 (31.3 ± 4.1), followed by VVD (4.2 ± 0.6), WC-2 (2.7 ± 1.1), FRQ (1.75 ± 0.6), and FWD-1 (0.17 ± 0.16), which had much lower expression (**Figure 5.22**). There were no differences between morning and evening expression.

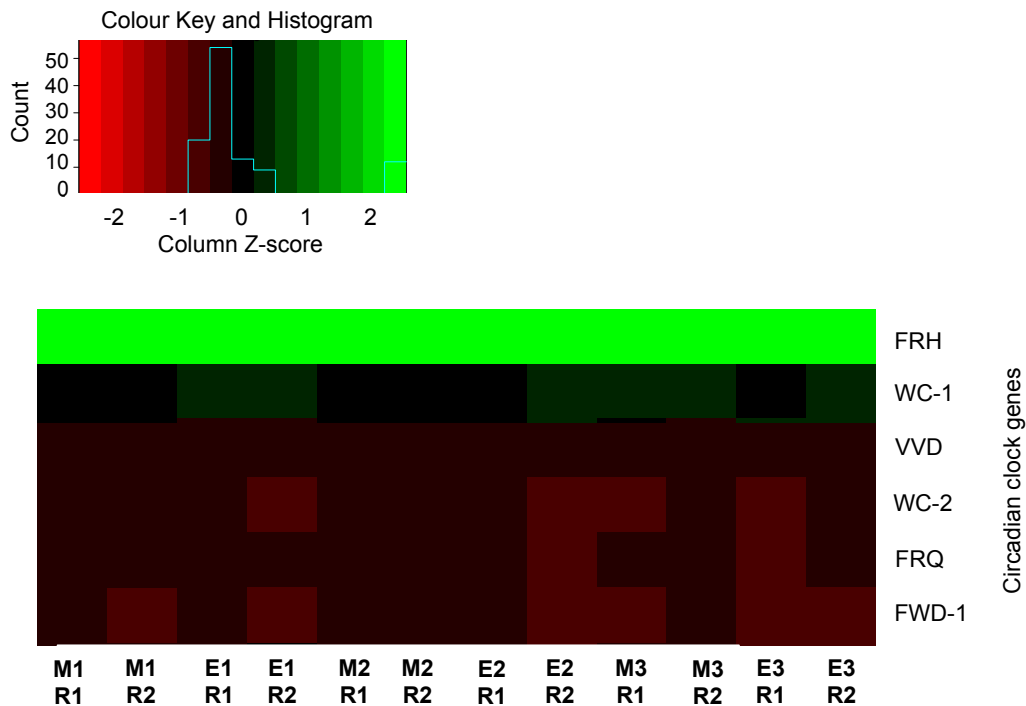


Figure 5.22 Circadian clock homologue read abundances (Column Z-score) in each meta-transcriptome, on each morning (M) and evening (E) 1 – 3, replicates (R) 1 and 2.

5.3.4 Meta-transcriptome analysis of root bacteria and archaea

A total of 1,120,000 subsampled functional reads were successfully aligned to the bacterial and archaeal database. The number of sequences per sample ranged between 76,130 and 107,041, with a mean of 93,333.

5.3.4.1 Bacterial and archaeal taxonomy from the meta-transcriptome

Altogether, there were 47 phyla, 85 classes, 234 orders, 346 families, 795 genera, and 1979 species. There were 64.6 % conserved reads across bacteria

and archaea, which were not assigned to a higher resolution because the lowest common ancestor method was used, which picks the lowest common ancestor when a gene is represented by more than one taxa homologue in the data set. From the remaining sequences, the phyla with the most active transcription (proportion of reads) included Bacteria (45.5 %), Proteobacteria (19.6 %), cellular organisms (10.0 %), Cyanobacteria (8.1 %), Firmicutes (2.7 %), Actinobacteria (02.4 %), Euryarchaeota (2.6 %), Bacteroidetes (2.1 %), Archaea (1.4 %), and Spirochaetes (1.0 %), (**Appendix Figure 5.8a**). The dominant active bacterial and archaeal classes, included Alphaproteobacteria (5.5 %), Gammaproteobacteria (4.1 %), Oscillatoriophyceidae (2.8 %), Actinobacteria (2.4 %), Clostridia (1.6 %), Betaproteobacteria (1.6 %), Spirochaetia (1.0 %), Chlamydiae (0.89 %), and Bacilli (0.87 %) (**Appendix Figure 5.8b**). The dominant active bacterial and archaeal orders included Actinomycetales (2.2 %), Chroococcales (2.1 %), Clostridiales (1.3 %), Rhizobiales (1.1 %), Spirochaetales (1.0 %), Chlamydiales (0.89 %), Rhodospirillales (0.91 %), Burkholderiales (0.95 %), Enterobacteriales (0.75 %), Bacillales (0.60 %) and Flavobacteriales (0.44 %). The dominant active bacterial and archaeal families, included Enterobacteriaceae (0.75 %), Spirochaetaceae (0.82 %), Clostridiaceae (0.55 %), Planctomycetaceae (0.48 %), Rhodospirillaceae (0.69 %), Burkholderiaceae (0.49 %), Nostocaceae (0.38 %), Mycobacteriaceae (0.16 %), and Streptomyetaceae (0.21 %). The dominant active bacterial and archaeal genera, included Cyanothecce (0.48 %), Streptomyces (0.19 %), Caldilinea (0.18 %), Thermomonospora (0.17 %), Acarylochloris (0.14 %), and Synechococcus (0.40 %) (**Appendix Figure 5.8c**). The most active species were related to *Prochlorococcus marinus* (0.38 %), *Trichodesmium erythraeum* (0.33 %), *Ruminococcus albus* (0.32 %), *Aciduliprofundum boonei* (0.28 %), *Rhodospirillum photometricum* (0.27 %), *Methanosaeta harundinacea* (0.24 %), *Desulfurivibrio alkaliphilus* (0.23 %), *Marinitoga piezophila* (0.23 %), *Traponema denticola* (0.22 %), *Caldilinea aerophila* (0.21 %), and *Thermomonospora curvata* (0.21 %) (**Appendix Figure 5.8d**).

5.3.4.2 Bacterial and archaeal community similarity in the morning and evening

When morning and evening samples were compared using ADONIS (Bray-Curtis dissimilarity), bacterial and archaeal communities were not significantly different, to $P < 0.1$, at any hierarchy (**Table 5.6**). **Figure 5.23** shows there was

some distance between the active morning and evening bacterial and archaeal communities, but it was a very slight shift, and many morning and evening community profiles overlap.

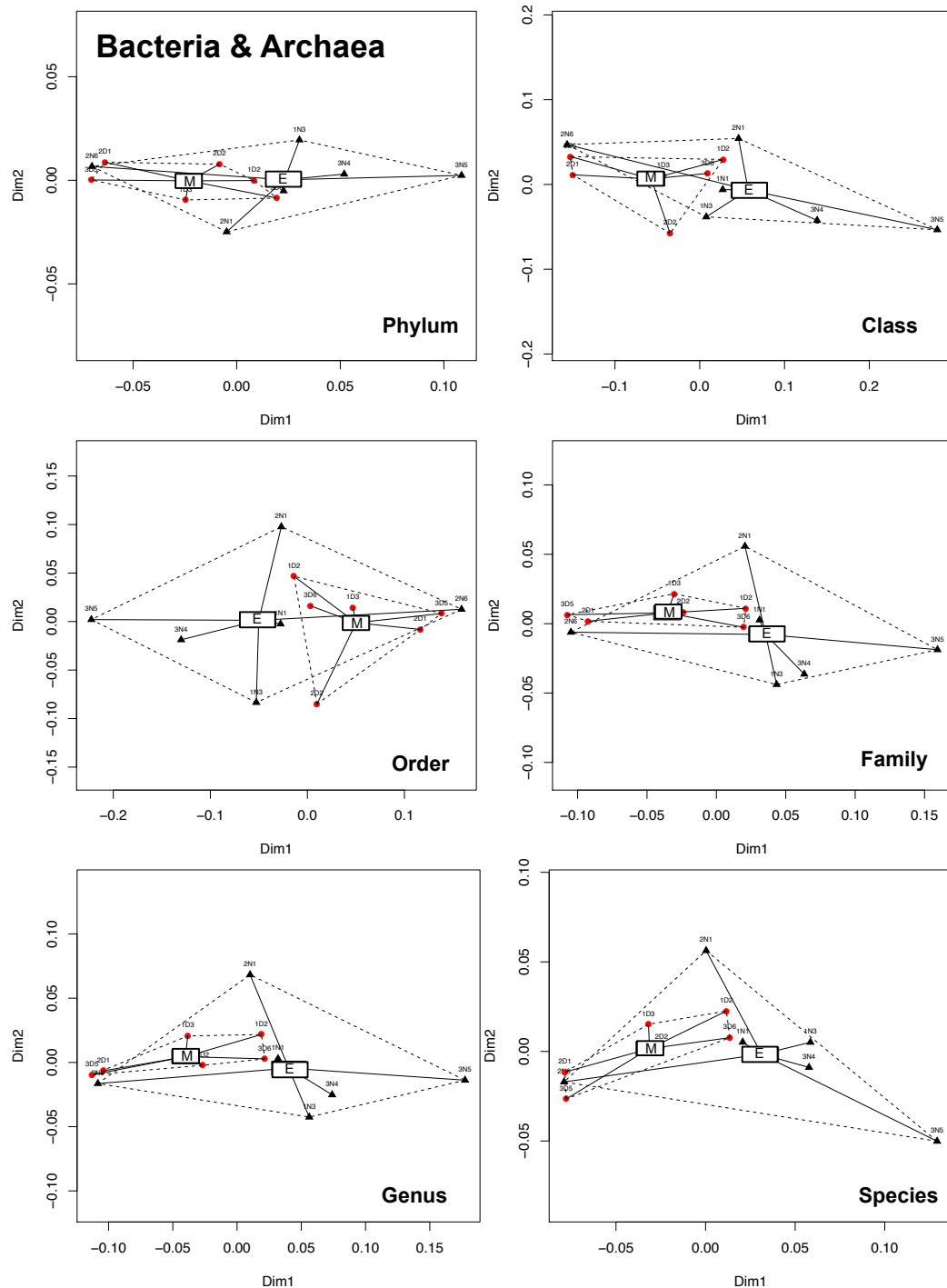


Figure 5.23 NMDS plot of bacterial and archaeal mRNA expression during the morning (M) and evening (E), where functional genes were assigned to phyla, class, order, genus, and species level. Points represent community profiles of each sample, where red circles are morning samples, and black triangles are evening samples. In data point labels, the first number represents the sampling day (1-3), and the final number is the replicate number.

Table 5.6 Summary statistics of the differences between morning and evening bacterial and archaeal gene expressions, assigned to varying levels of taxonomic classification, using ADONIS (Bray-Curtis dissimilarity).

Division	ADONIS (Bray Curtis)	
	r^2	P
Phylum	0.197	0.129
Class	0.195	0.128
Order	0.185	0.126
Family	0.178	0.124
Genus	0.170	0.114
Species	0.164	0.115

5.3.4.3 Bacterial and archaeal functional gene expression analysis

A total of 304,277 subsampled bacterial and archaeal sequences were functionally annotated from the meta-transcriptome. A number of functions were annotated with SEED; 27 at Level 1, 164 at Level 2, 599 at Level 3, and 1550 at Level 4 (**Appendix Figure 5.9a - d.**). At SEED Level 1 (**Appendix Figure 5.9a**), the most dominant functions involved carbohydrates (30.4 %), protein metabolism (18.7 %), amino acids and derivatives (9.8 %), stress response (8.2 %), respiration (6.3 %), virulence (4.1 %), cell wall and capsule (4.0 %), fatty acids, lipids, isoprenoids (3.3 %), cofactors, vitamins, prosthetics, pigments (2.2 %), and nitrogen metabolism (2.0 %) (**Appendix Figure 5.9a**). At SEED Level 2 (**Appendix Figure 5.9b**), the most dominant functions involved central carbohydrate metabolism (19.7 %), protein folding (8.3 %), protein biosynthesis (5.6 %), one-carbon metabolism (4.9 %), heat shock DnaK (4.6 %), Lysine, threonine, methionine and cysteine (4.6 %), protein degradation (4.1 %), and ATP synthases (3.7 %) (**Appendix Figure 5.9b**). At SEED Level 3 (**Appendix Figure 5.9c**), the most dominant functions included Entner-Doudoroff Pathway (10.1 %), Protein chaperones (5.1 %), Serine-glyoxylate cycle (4.6 %), Chaperone protein DnaK (3.9 %), Universal GTPases (3.8 %), TCA cycle (3 %), Proteolysis in bacteria ATP-dependent (2.7 %), Linker unit arabinogalactan synthesis (2.6 %), and Staphylococcal pathogenicity islands SaPI (2.6 %). Finally, at SEED Level 4 (**Appendix Figure 5.9d**), the most dominant functions included enolase (4.7 %), NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (4.6 %), chaperone protein DnaK (3.9 %), heat shock protein 60

family chaperone GroEL (2.6 %), and translation elongation factor 1 alpha subunit (2.3 %).

5.3.4.4 Bacterial and archaeal functional expression changes in the morning and evening

Bacterial and archaeal functional expression, between morning and evening, was not significantly different to $P < 0.1$, at any SEED level (**Appendix Table 5.2**), and there was only a small non-significant ($P > 0.1$) shift in the community profile clusters in the NMDS from morning to evening (**Appendix Figure 5.10**).

5.3.5 Targeted amplicon pyrosequencing of fungal ITS region

5.3.5.1 ITS fungal community pyrosequencing

There were a total of 306,132 raw sequences, before processing. Acacia denoiser discarded 118,135 sequences, corrected 30,698 sequences, and altogether retained 126,884 for analysis. After dereplication, UCHIME identified 3,270 chimeric OTUs, which were subsequently removed from analysis. There was a total of 42,352 usable sequences, and 6,283 OTUs. The UNITE database assigned taxonomy to 29,891 sequences, with a mean number of 1,661 (S.E.M \pm 188) per sample, and the remaining 12,461 sequences were unidentified. Altogether, OTUs were assigned to 257 taxa in the UNITE database sequences, with a mean of 55.2 taxa per sample (S.E.M \pm 7.2).

5.3.5.2 ITS fungal community pyrosequencing (DNA)

From DNA samples, there were a total of 29,835 usable sequences, with a mean of 4,973 (S.E.M \pm 144) sequences per sample. From these un-assigned sequences, there was a total of 4,800 OTUs, with a mean of 1,263 OTUs (S.E.M \pm 39.2) per DNA sample. The UNITE database assigned taxonomy to 20,587 DNA sequences, with a mean number of 3,431 (S.E.M \pm 101) sequences per DNA sample. The UNITE database assigned sequences to 210 taxa, with a mean of taxa 87.8 (S.E.M \pm 3.3) per DNA sample.

5.3.5.3 Comparison of morning and evening fungal communities (DNA)

In DNA samples, there was no difference between morning and evening communities, from both assigned (ANOSIM $r = -0.222$, $P = 0.800$) and unassigned communities (ANOSIM $r = -0.407$, $P = 1.000$) (**Table 1.**). There was no significant difference between morning and evening community structures (**Figure 5.8**). The abundance of fungal groups in the morning (M) and evening (E) were unidentified (mean relative abundance % \pm S.E.M, TTEST) ($M = 54.2 \pm 2.4$, evening = 48.5 ± 4.3 , $P = 0.31$), followed by Ascomycota ($M = 33.3 \pm 6.2$, $E = 39.3 \pm 9.6$, $P = 0.63$), Basidiomycota ($M = 8.2 \pm 5.9$, $E = 8.2 \pm 6.0$, $P = 1.00$), Glomeromycota ($M = 3.4 \pm 0.7$, $E = 4.0 \pm 0.8$, $P = 0.59$), Chytridiomycota ($M = 0.86 \pm 0.51$, $E = 0.06 \pm 0.06$, $P = 0.19$), and Basal ($M = 0.006 \pm 0.006$, $E = 0.02 \pm 0.01$, $P = 0.36$), none of which were significantly different from morning to evening (**Figure 9.**). The most dominant taxon from DNA samples was *Ophiosphaerella herpotricha* (mean relative abundance % \pm S.E.M) ($M = 15.1 \pm 6.4$, $E = 11.6 \pm 5.7$), followed by *Nectriaceae* sp. ($M = 11.6 \pm 4.7$, $E = 12.8 \pm 1.4$), *Exidia* sp. ($M = 6.2 \pm 6.3$, $E = 6.2 \pm 6.3$), and Lasiosphaeriaceae ($M = 3.6 \pm 2.1$, $E = 7.8 \pm 5.8$) (**Figure 10.**).

5.3.5.4 ITS fungal community pyrosequencing RNA

From RNA samples, there was a total of 12,517 usable sequences, with a mean of 1,043 (S.E.M \pm 65.6) per RNA sample. From these un-assigned sequences, there was a total of 2,488 OTUs, with a mean of 344 OTUs (S.E.M \pm 16.3) per RNA sample. The UNITE database assigned taxonomy to 9,304 RNA sequences, with a mean, of 775 sequences (S.E.M \pm 29.8) per RNA sample. The UNITE database assigned sequences to 139 taxa, with a mean of 38.8 OTUs (S.E.M \pm 3.25) per RNA sample.

5.3.5.5 Comparison of morning and evening fungal communities (RNA)

There was a significant difference between morning and evening active communities, in unassigned (ANOSIM $r = 0.259$, $P = 0.039$ *) (**Figure 8**), but not assigned sequences (ANOSIM $r = 0.207$, $P = 0.084$) (**Table 1**). The most dominant fungal group in RNA evening was the Glomeromycota (mean relative abundance % \pm S.E.M, TTEST) ($M = 26.3 \pm 3.6$, evening = 43.9 ± 5.8 , $P = 0.02$ *), whereas RNA morning was dominated by unidentified sequences ($M = 51.3 \pm$

3.5, $E = 28.2 \pm 5.2$, $P = 0.005$ *), however the remaining groups were not significantly different between morning and evening sampling times; Basal groups ($M = 0.1 \pm 0.05$, $E = 0.36 \pm 0.12$, $P = 0.07$), Ascomycota ($M = 17.3 \pm 1.9$, $E = 16.5 \pm 2.8$, $P = 0.81$), Basidiomycota ($M = 4.8 \pm 2.8$, $E = 9.5 \pm 6.2$, $P = 0.50$), and Chytridiomycota ($M = 0.17 \pm 0.06$, $E = 1.62 \pm 1.07$, $P = 0.21$) (**Figure 9**). The dominant taxa in the morning were very different from the evening. **Figure 10** shows that in the morning the most active taxa was *Ophiosphaerella herpotricha* (mean relative abundance % \pm S.E.M, TTEST) ($M = 21.1 \pm 5.6$, $E = 7.8 \pm 4.5$, $P = 0.89$), followed by *Glomus sp. 7 SUN-2011* ($M = 15.2 \pm 4.0$, $E = 7.3 \pm 2.4$, $P = 0.118$), and *Helotiales sp.* ($M = 3.3 \pm 2.2$, $E = 1.9 \pm 1.4$, $P = 0.575$). Whereas the dominant sequences in the evening belonged to *Paraglomus laccatum* (mean relative abundance % \pm S.E.M) ($M = 8.2 \pm 2.6$, $E = 31.1 \pm 8.4$, $P = 0.024$ *), followed by *Ophiosphaerella herpotricha* (as before), *Psilocybe sp.* ($M = 0.03 \pm 0.02$, $E = 6.3 \pm 6.4$, $P = 0.343$), and *Schizothecium sp.* ($M = 0.03 \pm 0.02$, $E = 3.5 \pm 3.5$, $P = 0.338$).

5.3.5.6 Comparison of ITS fungal communities from DNA and RNA

There was a significant difference between communities profiled using RNA and DNA, in both assigned (ANOSIM $r = 0.665$, $P = 0.001$ *) and unassigned communities (ANOSIM $r = 0.241$, $P = 0.028$ *) (**Table 5.7**). The difference between RNA and DNA is distinct in **Figure 5.24**, with RNA and DNA samples clustering separately. The dominant fungal phylum in both DNA and RNA samples was unidentified (mean relative abundance % \pm S.E.M, TTEST) (RNA = 39.8 ± 4.7 , DNA = 51.3 ± 2.6 , $P = 0.11$). However the next dominant fungal phylum in RNA samples was Glomeromycota, which was significantly higher than DNA (RNA = 35.1 ± 4.2 , DNA = 3.7 ± 0.5 , $P < 0.001$ *), and the dominant DNA group was Ascomycota, which was significantly higher than in RNA (RNA = 16.9 ± 1.6 , DNA = 36.3 ± 5.3 , $P < 0.001$ *). However the remaining groups were not significantly different between RNA and DNA; Basidiomycota (RNA = 7.1 ± 3.3 , DNA = 8.2 ± 3.7 , $P = 0.85$), Chytridiomycota (RNA = 0.9 ± 0.6 , DNA = 0.5 ± 0.3 , $P = 0.65$), and Basal (RNA = 0.23 ± 0.07 , DNA = 0.01 ± 0.01 , $P = 0.054$) **Figure 5.25**. The dominant OTU in RNA was *Paraglomus laccatum*, which was significantly higher than in DNA (RNA = 19.6 ± 5.5 , DNA = 1.5 ± 0.68 , $P = 0.024$ *), whereas in DNA the dominant taxa was *Ophiosphaerella herpotricha* (RNA = 14.4 ± 5.1 , DNA = 13.4 ± 6.0 , $P = 0.09$) (**Figure 5.26**).

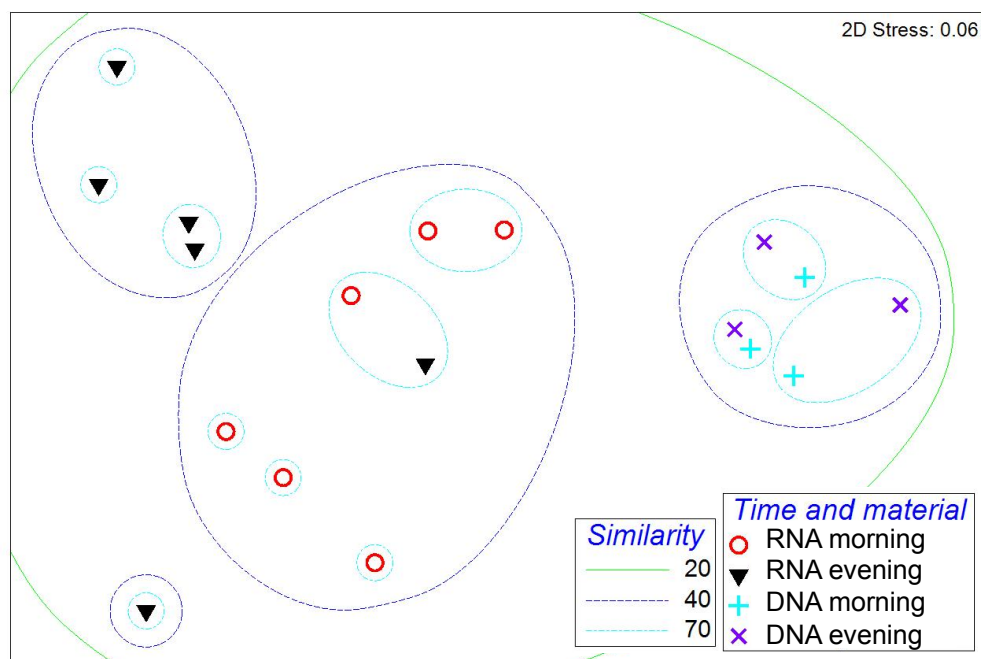


Figure 5.24 NMDS plot of fungal community Bray Curtis similarity, ITS region. Similarity lines are made from hierarchical cluster analysis, at 20 %, 40 % and 70 % group average similarity.

Table 5.7 Summary statistics for Analysis of Similarity (ANOSIM) of total fungal communities, between evening and morning, in DNA and RNA samples, and between DNA and RNA samples. Analysis of both Unassigned (all OTUs), and OTUs assigned to the UNITE database. * *P* values significant at 0.05 %.

Unassigned

Substrate	Comparison		<i>r</i>	<i>P</i>
DNA	Evening	Morning	-0.407	1.000
RNA	Evening	Morning	0.259	0.039 *
	DNA	RNA	0.241	0.028 *

UNITE

Substrate	Comparison		<i>r</i>	<i>P</i>
DNA	Evening	Morning	-0.222	0.800
RNA	Evening	Morning	0.207	0.084
	DNA	RNA	0.665	0.001 *

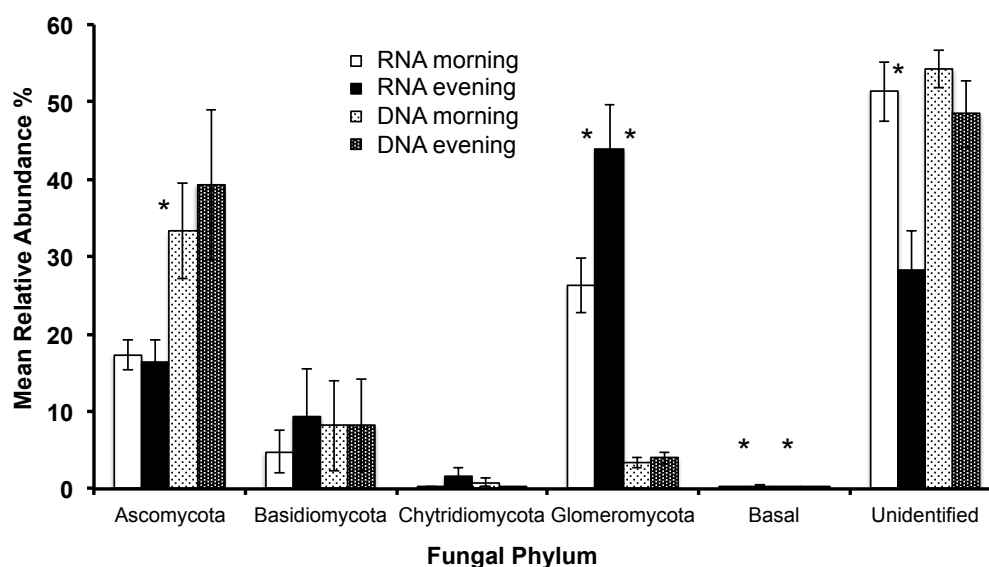


Figure 5.25 Mean relative abundance of fungal groups from ITS RNA, morning, RNA evening, DNA morning, and DNA evening. Where error bars represent S.E.M.

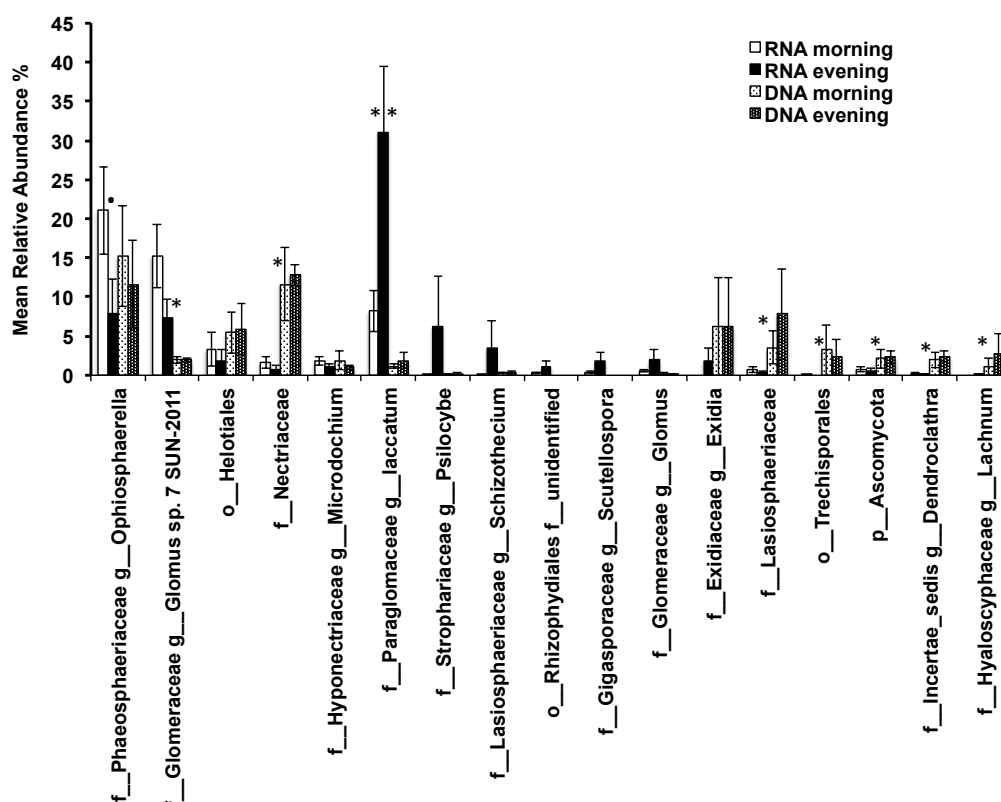


Figure 5.26 Mean relative abundance of fungal OTU (> 1 % of total abundance) from ITS RNA, morning, RNA evening, DNA morning, and DNA evening. Where error bars represent S.E.M, and significant TTEST *P*-values are indicated with ■ ($P < 0.1$), * ($P < 0.05$), between mean relative abundance of the bars it is positioned over, either morning and evening, or DNA and RNA. Taxa were named to the nearest, phylum (p) order (o), family (f) or genus (g), where identified.

5.3.6 Targeted amplicon pyrosequencing of AMF AML region

5.3.6.1 AMF community pyrosequencing

There was a total of 64,887 raw sequences, before processing. Acacia denoiser discarded 7,395 sequences, corrected 17,588 sequences, and altogether retained 51,566 for analysis. After dereplication, UCHIME identified 578 chimeric OTUs, which were subsequently removed from analysis. There was a total of 12,961 usable sequences, and 544 OTUs at 97 % similarity. The AMF clone database (from Chapter 3) assigned taxonomy to all of the usable sequences to class level, and 11,997 sequences to genus level. Altogether, OTUs were assigned to eight taxa in the AMF clone database.

5.3.6.2 AMF community pyrosequencing (DNA)

From DNA samples, there was a total of 2,964 usable sequences, with a mean of 449 (S.E.M \pm 35.4) sequences per sample. The AMF clone database assigned taxonomy to all 2,964 DNA sequences to class level, and 2,502 to genus level, with a mean number of 449 (S.E.M \pm 35.4) sequences per DNA sample. The AMF clone database assigned sequences to thirteen taxa, with a mean of 11.0 taxa (S.E.M \pm 0.73) per DNA sample.

5.3.6.3 Comparison of morning and evening AMF communities (DNA)

There was no difference between community structure in the morning and evening, in DNA, from both taxonomy assigned (ANOSIM $r = -0.269$, $P = 0.90$) and unassigned communities (ANOSIM $r = -0.519$, $P = 1.00$) (**Table 5.8**). The similarity between morning and evening is evident in **Figure 5.27**, as morning and evening samples do not cluster separately. At species level (97 %), the dominant taxa were *Glomus iranicum*, *Paraglomus laccatum* and *Glomus other* (unidentified). There were no significant differences between AMF species in the morning and evening (**Figure 5.28**).

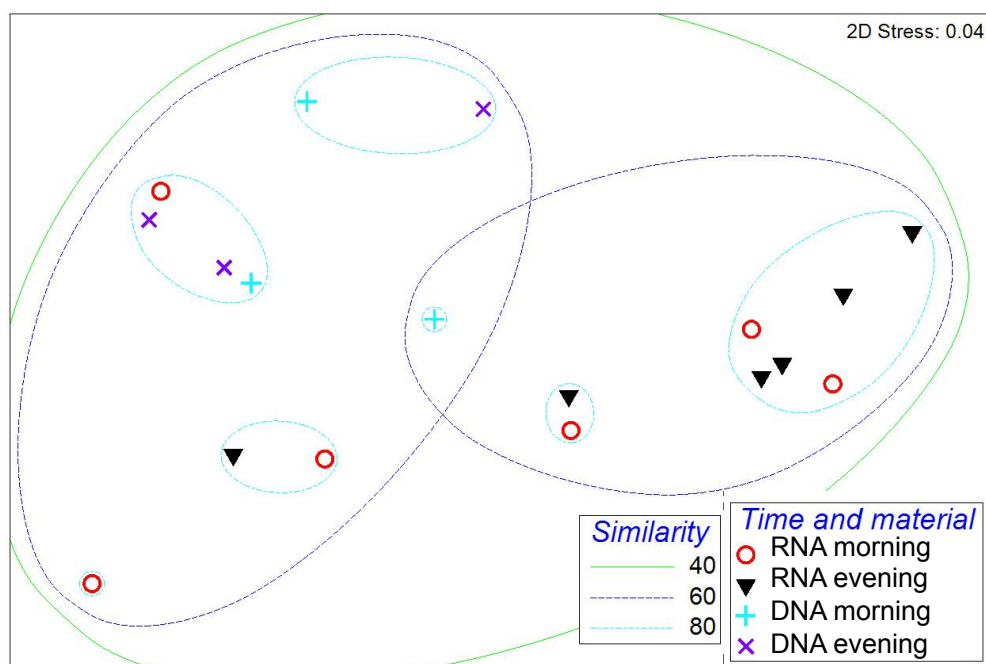


Figure 5.27 NMDS graph of Bray Curtis similarity, of AMF clone database assigned OTUs (Species level), from 18S rRNA from DNA and RNA, during the morning and evening. Similarity lines created using hierarchical clustering.

Table 5.8 Summary statistics for Analysis of Similarity (ANOSIM) of AMF communities, between evening and morning, in DNA and RNA profiles. * *P* values significant to 0.01 %.

Unassigned

Substrate	Comparison		<i>r</i>	<i>P</i>
DNA	Evening	Morning	-0.519	0.900
RNA	Evening	Morning	0.054	0.210
	DNA	RNA	0.595	0.001 *

AMF Clone db

Substrate	Comparison		<i>r</i>	<i>P</i>
DNA	Evening	Morning	-0.269	1.000
RNA	Evening	Morning	0.019	0.300
	DNA	RNA	0.265	0.02 *

5.3.6.4 AMF community pyrosequencing (RNA)

From RNA samples, there was a total of 10,267 usable sequences, with a mean of 855 (S.E.M \pm 103.4) per RNA sample. The AMF clone database assigned taxonomy to all 10,267 sequences to class level, and 9,495 sequences to genus

level. The AMF clone database assigned sequences to fifteen taxa, representing eight species, with a mean of 10.8 OTUs (S.E.M \pm 0.53) per RNA sample.

5.3.6.5 Comparison of morning and evening AMF communities (RNA)

There was not a significant difference between morning and evening active communities, from either taxonomy assigned (ANOSIM $r = 0.019$, $P = 0.30$) or unassigned communities (ANOSIM $r = 0.054$, $P = 0.21$) (**Table 5.8**). In addition, morning and evening profiles did not cluster separately (**Figure 5.27**). At species level (97 %), the dominant taxa were *Paraglomus laccatum*, *Glomus other* (unidentified), and *Glomus iranicum*. There were no significant differences between AMF species in the morning and evening (**Figure 5.28**).

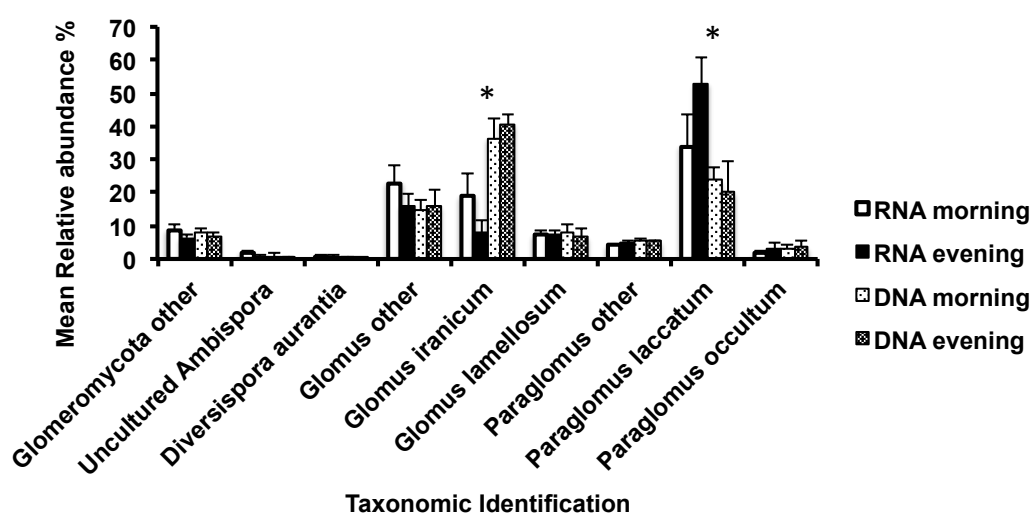


Figure 5.28 Mean relative abundance all AMF taxa in the 18S rRNA library, at species level 97 % similarity to AMF clone database, where * ($P < 0.05$).

5.3.6.6 Comparison of AMF communities from DNA and RNA

There was a significant difference between community structures from RNA and DNA samples, in both assigned (ANOSIM $r = 0.265$, $P = 0.02$ *) and unassigned communities (ANOSIM $r = 0.595$, $P = 0.001$ *) (**Table 5.8**). The difference between RNA and DNA communities is clear for most samples, as RNA and DNA profiles cluster separately (**Figure 5.27**). At species level (97 % similarity), *Glomus iranicum* was significantly (mean relative abundance, SEM, TTEST) higher in DNA than RNA samples (RNA = 13.3 (\pm 4.2), DNA = 38.4 (\pm 3.3), $P = 0.013$ *), and *Paraglomus laccatum* was significantly higher in RNA than DNA samples (RNA = 43.2 (\pm 6.7), DNA = 22.0 (\pm 4.53), $P = 0.0498$ *) (**Figure 5.28**).

5.4 Discussion

There are a number of major findings from this work, including that roots and rhizosphere microbes, particularly fungi, have circadian patterns of activity and function. Another major finding is the possible circadian interplay between timing of plant defenses, with pathogen and symbiotic activity, and of circadian uptake of nutrients in plants and fungi, which could have major implications for crop management and plant breeding. Presented is the simultaneous characterization of plant, fungal, other eukaryotic microbes, bacterial, archaeal, structure and function, utilizing meta-transcriptome technology to its full, which has brought forward the understanding of temporal dynamics in the rhizosphere.

5.4.1 *Miscanthus giganteus* functional differences from morning to evening

M. giganteus roots had significant shifts in function from morning to evening, from a broad pathway down to individual genes, which is a major finding, as there is very limited knowledge of plant circadian patterns in the roots, and even less about circadian interactions in the environment. The environment seemed to play a large role in the circadian patterns of gene expression, as the dissimilarity between morning and evening was more significant when the interaction between days was included. Both time and the environment affected downstream processes, in particular nitrogen metabolism, which is thought to be linked to circadian regulation (Gutierrez, *et al.*, 2008). These results are significant in developing our understanding of the way environment and circadian regulation interact.

5.4.1.1 Cluster 5&6

From the functional gene DEGs, cluster 5&6 had the strongest diurnal oscillations, with higher expression of enriched genes involved with response to nitrate, nitrogen transport, acyl transferases, and response to wounding in the evening, which suggests it could be important to upregulate nitrogen and defense pathways in the evening, to adapt to environmental changes.

5.4.1.2 Plant defense genes

A significantly enriched number of defense genes were more active in the evening, including response to chitin and jasmonic acid, which could be the reason Ascomycota phyla, in particular the pathogenic *Ophiosphaerella herpotricha*, were less active, and symbiotic fungi were more active, in the evening, as discussed earlier. Some plant-pathogen interactions have been found to be affected by light and circadian patterns (Roden and Ingle, 2009). However, previous research has shown that plant phyllosphere defenses, for example against downy mildew, are more active in the morning, involving regulation by *CCA1*, (Wang, *et al.*, 2011b) and *Pseudomonas syringae* (Bhardwaj, *et al.*, 2011; Ingle and Roden, 2014). Therefore, enhanced plant root defenses in the evening are a novel discovery and more research needs to address whether this phenomenon is plant, tissue, or pathogen specific.

5.4.1.3 Plant circadian nutrient uptake

Response to nitrate and nitrogen assimilation were significantly enriched in the evening, which strongly suggests that nitrogen uptake activity was greater in the evening. Nitrate assimilation may be regulated by, and be a regulator of, the circadian master clock control gene *CCA1* in roots. A nitrogen-responsive gene network was found to be involved in circadian clock regulation, whereby N-nutrient input triggered a change of *CCA1* and *LHY* expression, leading to the assimilation of N, and the synthesis of amino acids (Gutierrez, *et al.*, 2008). However, this has not been shown in the field before. N was found to be excreted more from leguminous plant roots at night, however uptake of NO_3^- was the same during the day and night (Wacquant, *et al.*, 1989). However, leguminous plants are likely to have an excess of N, due to their symbiotic relations with nitrogen fixing bacteria. The evidence presented here indicates that roots are actively taking up and metabolising more nitrogen into amino acids in the evening, which could be circadian regulated.

High affinity K^+ transporters were significantly higher in all three nights than the day, which suggests K uptake is also more active at night. Perhaps there is more nutrient uptake activity in the evening, as during the morning and throughout the day, plants direct their energy towards fixing carbon, to maximize photosynthesis during sunlight hours. Therefore, it could be more efficient to

assimilate nutrients in the evening, when photosynthetic machinery is less active.

If nutrient availability and deficiency can train the central component of the root circadian clock, this could also be a response to changes in nutrients made available by microbes. Symbiotic microbes were more active in the evening, which are known to exchange nutrients for plant carbon (Smith and Read, 2008). Perhaps the theory of reciprocal rewards, which ensures cooperation from both partners to form a stable mutualism (Kiers, *et al.*, 2011), works on a diurnal cycle. Whereby, microbes make more nutrients available in the evening, because there are more carbohydrate rewards at that time, and in response plants train their root circadian clock to the availability of nutrients, to anticipate the period in the day when nutrients will become available. This could make downstream expression of nutrient uptake more efficient, as a major function of the roots is to uptake nutrients, similar to the way the phyllosphere circadian clock is trained by light to anticipate sunrise to maximize efficiency of the photosynthetic period (Covington, *et al.*, 2008). The fungal meta-transcriptome showed that nitrogen assimilation was higher in the morning, however, the dominant fungus was a root pathogen, and was likely to dominate the functional analysis, over the under represented Glomeromycota. This concept has not been explored in the literature, and is an exciting new area of research, which needs to be developed.

5.4.1.4 Cytochrome P450

Cytochrome P450 genes were more active in the evening, and are involved with the biosynthesis of the majority of the known secondary compounds which react to the changing environment, such as by redirecting metabolism to defense or communication (Nielsen and Moller, 2005). P450s are differentially expressed in nutrient deficient plants, to adapt and cope with the stress, by diverting metabolism (Cai, *et al.*, 2013). At least ninety-eight cytochrome P450 enzymes have been found to be reporters for the circadian clock in plant leaves, including those involved with the synthesis of glucosinolate, brassinosteroid, and phenylpropanoid (Bancos, *et al.*, 2006; Pan, *et al.*, 2009). Cytochrome P450s are also linked to glucosyltransferases in the roots, involved in gibberellin synthesis (Kim, *et al.*, 2014), and glutathione-S-transferases, involved in the nitrogen responsive circadian network described previously (Appiah-Opong, *et*

al., 2008), which were both more active at night. However previous studies have analysed phyllosphere tissue, and most cytochrome P450s peak just before subjective dawn (Pan, *et al.*, 2009). Whereas, in this study of the rhizosphere, all of the cytochrome P450s were higher in the evening. This may be due to a difference in functions of the tissues; leaves prepare for photosynthesis to maximize efficiency during the day, whereas roots appear to be more active in the evening, for many functions including nutrient uptake and defense.

5.4.1.5 Plant hormones

A terpenoid cyclase, involved in the brassinosteroid gibberellin biosynthesis was differentially expressed on all days and was very abundant, which may have been controlled by one of the diurnally expressed cytochrome P450s (Nielsen and Moller, 2005), which were also differentially expressed on all days. Brassinosteroids promote stress resistance, growth and photomorphogenesis, and their levels are tightly controlled, by negative feedback. The accumulation of brassinosteroids peak in the middle of the day in *Arabidopsis* shoots (Bancos, *et al.*, 2006). However, in this experiment, they were found to be higher in the evening. Response to auxins, jasmonic acids, stress, and abiotic stress, were also differentially expressed. Multiple plant hormones, including ethylene (Finlayson, *et al.*, 1999), have been linked to stress responses, and many stress responses have been linked to the circadian clock control (Covington, *et al.*, 2008).

5.4.1.6 Clusters 1, 2, 3, and 4

Clusters 1, 2, 3, and 4, had significantly oscillating genes, but the patterns were more variable and less consistent. However, it was interesting that in cluster 2, secondary cell wall biogenesis activity was very high on the first and third night, but was very low on the second night. The reduced growth on the second night may have been a response to the previous cold night, so that other functions may have been prioritised above secondary cell wall biogenesis on the second night.

5.4.1.7 Uncharacterised proteins

There were a number of uncharacterized proteins (not presented), which were differentially expressed. These could have a regulatory or downstream role in the circadian clock, and warrant further investigation.

5.4.1.8 Plant circadian clock genes

Homologues of all eight circadian clock genes were identified in the *Miscanthus* root transcriptome, which means circadian regulation was active in the roots and could be responsible for the diurnal patterns in gene expression. *CCA1* and *LHY* homologues were significantly enriched in the morning, similar to the findings of James, *et al.* (2008). *LUX*, *PRR7* and *PRR9*, *GI*, and *TOC1* were higher in the evenings, which was expected of *LUX*, *GI*, and *TOC1* (James, *et al.*, 2008), although *LUX* had very low expression. *PRR7* and *PRR9* were previously found to be morning components, but their expression is promoted by *CCA1* and *LHY*, and may have had higher expression later in the morning (Hsu and Harmer, 2014). *CCA1*, *LHY*, *GI*, *PRR7* and *PRR9* were the most highly expressed in the roots, and were likely to be the main components of the circadian regulation in this system.

5.4.2 Environmental factors affecting the *Miscanthus* transcriptome

Overall, 1.1 % of genes were significantly different between mornings and evening, which is relatively low compared to studies in the phyllosphere. The root circadian clock is thought to be a smaller version of the phyllosphere clock (James, *et al.*, 2008), as there is no light, and it is thought to be less affected by aboveground environment changes. However, a number of environmental factors, such as moisture, temperature, and carbon fixation had significant effects on gene clusters, which may have affected circadian regulation of genes. Whereas, in lab systems, these variables are tightly controlled, so that temperature and light have the same pattern everyday, so circadian patterns appear more strongly. Therefore, there may be many more genes in the roots, which are circadian regulated, but they are more affected by the environment. In addition, not all circadian genes peak or trough at the 8 am and 8 pm, and other experiments measured significance of gene oscillation by correlation over more time points. For example, in *Arabidopsis*, 13.7% of shoot genes were scored

rhythmic, but only 3.2% of root genes were (James, *et al.*, 2008). Therefore, more genes may have been under circadian regulation, but were not noticed between these narrow time points, and strict levels of significance, which required genes to be two fold higher to qualify as significantly different, and must overlap between two models. However, with these strict criteria, we are confident the DEGs identified in the analysis were biologically significant.

5.4.2.1 Carbon fixation, respiration, and temperature

Over the three sampling days, GPP peaked at midday and respiration peaked in the night, which shows there were typical daily cycles of carbon fixation in the day and respiration by plants and microbes in the night. However, there was no drop in temperature the night before the first morning and a rapid decline in temperature on the first night, which coincided with low carbon fixation in the first day, and higher carbon fixation on the second day (**Figure 5.7**). The temperature fluxes may have lead to some of the differences in plant and microbial activity between days, due to the changing sugar levels available in the roots. For example, fungal communities of mornings clustered closer with the previous evening than the following evening, in the PCA plot **Figure 5.10** which may have been caused by differences in sugar availability accumulated over the day and environmental conditions affecting plant gene expression, as seen in the WCGNA (**Figure 5.17**).

5.4.3 Microbial communities in the roots of *Miscanthus giganteus*

The microbial community in the roots was very diverse, with over 136 microbial phyla detected, mostly eukaryotes, whereas other published meta-transcriptomes have been overwhelmed by bacterial reads (Turner, *et al.*, 2013). This is the first root meta-transcriptome to demonstrate the power of the eukaryote selection technique to characterize the incredible diversity within roots. Among the microbial eukaryotes, fungal populations were clearly very dominant in the meta-transcriptome, particularly the Ascomycota and Basidiomycota. However, despite selection for eukaryote mRNA, there was still a large proportion of bacterial reads, which indicates the roots may have been dominated by bacteria, and the selection method cannot eliminate all non-eukaryote reads. Around 3 million, out of 180 million, reads were identified as microbial, meaning the large majority of sequences were plant. The dominance

of plant sequences was expected, as samples were taken from roots which also have eukaryote mRNA, but this may be a limiting factor for other microbial researchers, if plant transcription is of no interest.

5.4.4 Fungi

5.4.4.1 Fungal communities and transcriptomes in the roots of *Miscanthus giganteus*

The most dominant fungal taxon in the ITS region analysis, was most related to the Ascomycota *Ophiosphaerella herpotricha*, a root pathogen, which was not found in the meta-transcriptome. However, the dominant taxon in the meta-transcriptome was *Parastagonospora nodorum*. The meta-transcriptome database was made from functionally annotated reads, mainly from published genomes. Therefore the dominant Pleosporineae fungi in the meta-transcriptome, *P. nodorum*, was likely to be the same fungus as *O. herpotricha* in the ITS analysis, particularly as over three million reads aligned to the *P. nodorum* genome, and it was the closest published genome related to *O. herpotricha*. There is a much larger diversity of annotated ITS regions, than functional genes, and the ITS region provided a more robust analysis for taxonomic evaluation (Bellemain, *et al.*, 2010), particularly at species level. However, the meta-transcriptome was useful in providing a non-selective overview of entire fungal populations and functions, which highlights the importance of creating amplicon sequences libraries in parallel to meta transcriptome analysis, and the potential to use meta-transcriptomes to pull out individual transcriptomes.

The Basidiomycota were also highly active in the roots, in ITS DNA, ITS RNA, and meta-transcriptome analyses, of fungal community structure and the genome alignment. The transcript alignment to the *Coniophora puteana* genome, revealed that even though Basidiomycota were the second most dominant phyla, and *C. puteana* was one of the most abundant species, it had over eighteen times less transcripts than *P. nodorum*, and four times less transcripts than *R. irregularis*. It is not clear what the functional significance of Basidiomycota was to *M. giganteus*. Many Basidiomycota often form ectomycorrhiza with tree and woody species, but *M. giganteus* does not form ectomycorrhiza. *Coniophora puteana* is a wood-rot fungus, which had its

genome published to understand its wood degrading activity (Martinez, *et al.*, 2009), and could be a saprophyte on the roots. However, the dominant Basidiomycota may not have been *C. puteana*, it may be the closest published genome to another Basidiomycota relative.

The fungal community appeared to vary greatly depending on molecular starting material and analysis. Ascomycota fungi dominated ITS DNA and the meta-transcriptome analysis, where Glomeromycota only represented a tiny proportion of annotated reads from ITS DNA (3.7 %). However, Glomeromycota dominated ITS RNA, around eleven Glomeromycota taxa were found in 18S rDNA and rRNA, and *Paraglomus laccatum* was found to be dominant in 18S and ITS RNA targeted amplicon libraries. There seems to be massive discrepancy between communities of Ascomycota and Glomeromycota when profiled using DNA and RNA, which is difficult to explain. In addition, only a small proportion of the meta-transcriptome was annotated with Glomeromycota (2,226 reads, 0.08 %), whereas an enormous number of 742,761 reads were aligned to the *Rhizophagus irregularis* genome. The difference between meta-transcriptome and transcriptome analyses may be that a large proportion of Glomeromycota genes are not annotated, as there is only one Glomeromycota genome (*R. irregularis*), of which only 62 % of genes had similarity to known domains or proteins, and there were many Glomeromycota specific genes (Tisserant, *et al.*, 2013). In addition, the large majority of the fungal meta-transcriptome could not be taxonomically annotated (55.3 %) or were not annotated past fungi (21.2 %), even though the database was made entirely from all published fungal non-redundant genes. The un-annotated fungal genes are likely to be conserved functions across fungal lineages, and therefore difficult to distinguish between, particularly using the lowest common ancestor method for annotation. Therefore, either most of the Glomeromycota reads were amongst the conserved and unidentified genes, which underestimated their abundance in the fungal meta-transcriptome analysis, or a large number of conserved genes were aligned to the Glomeromycota, which overestimated their abundance in the *R. irregularis* transcriptome.

There were also a large number of unidentified fungal reads in the ITS DNA, ITS RNA, and meta-transcriptome. The high proportion of unidentified fungi highlights the enormous diversity of fungi yet to be characterized, as only 70,000

out of the estimated 1.5 million fungal species are currently described (Hawksworth, 2001).

Meta-transcriptome technology is still very novel and experimental, and will undoubtedly improve as databases grow. The research presented here highlights the need for more fungal genomes to be sequenced and annotated, particularly Glomeromycota genomes, to realize the full potential for meta-transcriptomes to simultaneously characterise structure and function of microbial communities, and presented are the methods and analysis available to realize this potential.

5.4.4.2 Fungal activity in the morning and evening

There was a large shift in active fungal communities, between morning and evening, at order down to species level, which is a major finding, as it was previously not known whether microbial activity in the rhizosphere is affected by time of day. Activity of symbiotic Glomeromycota, particularly *Paraglomus laccatum* in 18S rRNA and ITS RNA, and Basidiomycota in ITS RNA, both almost doubled in the evening, and in the meta-transcriptome, Glomeromycota, and Basidiomycota groups, Agaricomycetes, Agaricales, and Coniophoraceae and *C. puteana*, were 26.2 %, 20 %, 65.5 %, 39.5 %, twelve times more active, in the evening than the morning, respectively. Reads homologous to an insect and an amphibian pathogen, *Metarhizium anisopliae* and *Batrachochytrium dendrobatidis*, also had higher activity in the evening, by 20.0 % and 16 %, respectively. Whereas, the activity of the fungi homologous to pathogenic Ascomycota fungus *Ophiosphaerella herpotricha* was three times higher in the morning than the evening in ITS RNA. Furthermore, in the meta-transcriptome, Dothideomycetes, Pleosporales, Pleosporinae, Phaeosphaeriaceae, and *P. nodorum* were all over three times more active in the morning than the evening. Other groups including Chytridiomycota, Microsporidia, Leotiomycetes, Eurotiomycetes fungi, were also more active in the morning than the evening. These results suggest some root pathogenic and saprotrophic fungi were more active in the morning and symbiotic fungi more active in the evening. The pathogenic fungi may be controlled in the evening, as plant root defenses were shown to be higher in the evening. Whereas symbiotic fungi may be able to avoid the plant defense responses in the evening, as the *R. irregularis* genome annotation revealed a lack of factors which are known to trigger plant defenses

(Tisserant, *et al.*, 2013). This would allow them to flourish in the evening, when there is less competition from pathogens and higher amounts of newly fixed carbon available in the roots. The higher symbiotic activity in the evening, and plant's control of pathogen activity, may be a co-evolutionary processes between plants and symbionts, to enhance exchanges, particularly as plant roots redistribute 95 % of their carbon before dawn (Graf, *et al.*, 2010). If these results are replicated in other crops, this could have major implications for crop management and genetic breeding of crops, such as the optimisation of fungicide application to the morning when pathogens are more active, or the upregulation of defense genes in the evening, to control pathogens and encourage symbiotic relations. Different fungi had different circadian patterns, and therefore the diurnal sampling of more crops roots is an area of research which needs to be explored further, to optimize the control of pathogens and encourage symbiotic fungi, to improve crop yields.

5.4.4.3 Fungal function in the roots of *Miscanthus giganteus*

A large number of fungal functions were identified in the meta-transcriptome, which is a major achievement, as this is the first description of root fungal function using a meta-transcriptome. The analysis showed that the main functions of fungi were involved with metabolism of proteins, RNA, nitrogen, aromatic compounds, carbohydrates, cell walls and DNA, and stress response. Nitrogen metabolism was particularly interesting because it involved all stages in nitrogen assimilation, including organic-N breakdown from urea, the conversion of NO to nitrate (flavoheмоprotein), assimilation of ammonia, and then catabolism of amino acids, glutathione, RNA, DNA, and proteins. Therefore the nitrogen cycle was extremely important, and a dominant feature, of fungal function. Stress response was also a dominant feature, mainly involved with oxidative stress.

5.4.4.4 Fungal functional differences from morning to evening

Fungi had significantly different functional profiles in the morning and evening, a trend evident from analysis of broad functional groups to individual genes, which is a major finding, as little is understood about diurnal functions of fungi in the environment, and particularly diurnal patterns in the rhizosphere. Many functions were higher in the morning, which could be due to an interaction with the plant,

as the plant transcriptome analysis showed that plant defenses, including response to chitin, were higher in the evening. However, the functional shift from morning to evening was stronger than the taxonomic shift, which could mean that the community stays relatively stable, but they just shift their functions. The fungal functions with the largest dissimilarity, being more active in the morning, were the nitrogen assimilation functions; metabolism of nitrogen, protein, RNA, and amino acids, and carbon assimilation functions; cell wall and capsule, carbohydrates, fatty acids, lipids, and isoprenoids. However, nitrogen and carbon metabolism are higher in the plant roots in the evening (Haydon, *et al.*, 2011), which means there could be a lag period between plants assimilating carbon and nitrogen, to then releasing it in the morning as root exudates or rhizodeposition (byproducts of roots growth) (Dennis, *et al.*, 2010), when rhizosphere fungi such as saprophytes can utilize the plant products (Leake, *et al.*, 2006). The only function more active in the evening was respiration, which was supported by the measurements of CO₂ fluxes, which was the first indication that plant and microbial respiration was higher in the evening.

5.4.4.5 Fungal circadian clock genes

Homologues of all the fungal circadian genes were found in the meta-transcriptomes, which is particularly interesting in the rhizosphere, as there is no light to stimulate blue-light receptors and temperature gradients are much lower than aboveground, to train circadian clock components. Rodriguez-Romero, *et al.* (2010), suggested fungal light receptors are switched on by light, to adapt to exposure to air, and to train the circadian clock. However, the light sensing circadian components were active in the rhizosphere, and could be the regulators of the differences found in fungal taxonomy and function between morning and evening. *FRH* and *WC-1* are likely to be the most important regulators in this system, as they were overwhelmingly the most abundant circadian clock homologues detected in the roots, which is likely due to the conservation of this genes across most fungal lineages (Salichos and Rokas, 2010). There were no differences between the morning and evening expression of these circadian clock homologues, which may have been due to the low number of reads. Alternatively, fungal clocks may be dampened underground, similar to the hypothesised smaller circadian clock in plant roots (James, *et al.*, 2008). Aboveground conditions fluctuate more in light and temperature, than underground conditions, which may have a dampening effect on the

underground fungal clocks compared to aboveground clocks. Or the root clock may be dampened compared to the controlled lab systems circadian experiments are normally carried out under. There could also be other factors, which are more important to fungal diurnal activity, such as environmental factors, or response to plant defenses, rhizodeposits, and symbiotic factors. However this research has proven the use of meta-transcriptome in searching for multiple functions, and identified a number of environmental circadian clock homologues, which were active in the rhizosphere of all twelve samples.

5.4.5 Bacteria and archaea

5.4.5.1 Bacterial and archaeal community structure and function in the roots of *Miscanthus giganteus*

Eukaryote mRNA was specifically selected and enriched experimentally, therefore the bacterial and archaeal communities detected reflects only a portion of their total community, which must be considered while interpreting these results. Despite being selected against, and with half the number reads compared to fungi, there were three times more bacterial and archaeal taxa, than fungi, which were mostly bacteria, which means bacteria are likely to have dominated the roots, and the library had it not been for eukaryote selection. Similar to the fungal taxonomy analysis, over half of reads could not be confidently identified past kingdom, which means there were a large number of conserved genes across bacterial phyla. Bacterial communities were dominated by Proteobacteria, Cyanobacteria, Firmicutes, Actinobacteria, and Bacteroides, which was similar to root communities of wheat, oat, and pea roots (Turner, *et al.*, 2013), although there were more Cyanobacteria, and Spirochaetes in *M. giganteus* roots. A number of groups were found which are known to have interactions with plants such as the Proteobacteria, Burkholderias and Rhizobiales (Lu, *et al.*, 2006). A number of nitrogen fixing bacteria, and cyanobacteria, which fix nitrogen and carbon, were found, including Rhizobiales, Nostocaceae, *Trichodesmium erythraeum*, and *Rhodospirillum photometricum*. These nitrogen fixers may be encouraged in *M. giganteus* fields, as nitrogen fertilizer is not needed (pers com Paul Carver, New Energy Farms), and research has shown adding nitrogen fertilizer to soils reduces the diversity and abundance of these bacteria (Berthrong, *et al.*, 2014). However, other reads related to species such as *Aciduliprofundum boonei*, an obligate

thermoacidiphilic archaea from deep sea vents (Schouten, *et al.*, 2008), may just be related to species with published genomes.

5.4.5.2 Bacterial and archaeal function in the roots of *Miscanthus giganteus*

The functional profile of bacteria and archaea was extremely diverse, with 1,550 L4 functions, compared to fungi, which only had 131 L4 functions. The abundance of bacterial functions may partly be due to the publication of more bacterial functional genes, but it is also likely that the bacteria are more functionally diverse than fungi (Damon, *et al.*, 2012; Urich, *et al.*, 2008). A number of the major functions in bacteria had similar proportions to fungal functions, including cell wall and capsule, fatty acids, and stress response. However, the dominant bacterial function in the roots was carbohydrates (30 %), which was massive compared to fungal carbohydrates (1.2 %). Virulence and amino acids were also higher in bacteria. In addition, protein metabolism, RNA metabolism, and respiration, had much lower proportions in bacterial communities, than fungi. Nitrogen metabolism in bacteria was lower than fungi, despite a number of nitrogen fixing bacteria found in the roots. However, the abundances of genes may have been altered during eukaryote mRNA enrichment.

5.4.5.3 Bacterial and archaeal differences from morning to evening

There were no significant differences in bacterial and archaeal morning and evening expression of taxonomy or function, which was a surprise as fungi had diurnal shifts. There was a small non-significant shift, which was noticeable on the NMDS plots, and the top nine bacteria phyla in the SIMPER analysis were slightly higher in the morning. However, these were very small shifts and were not significant. It was surprising that there were bacteria which could potentially fix nitrogen in the roots, for example cyanobacteria or the proteobacteria *Rhodospirillum photometricum*, but nitrogen metabolism was not a major influence in the bacterial SIMPER analysis, unlike in fungal communities. Carbohydrates were the most important diurnal dissimilarity in bacterial communities, which were higher in the morning. Carbohydrates are likely to be influenced by diurnal patterns aboveground, particularly in photosynthetic organisms, for example, cyanobacteria, which were a dominant group in the

roots. Some cyanobacteria spp. are known to have ~30 % of their transcriptome under circadian clock regulation, in controlled systems, which is important for maximizing efficiency of photosynthesis (Ito, *et al.*, 2009). However the diurnal shift in cyanobacteria and carbohydrates was much less pronounced underground in field conditions, and non-significant. Little is understood about the circadian regulation of bacteria, aside from photosynthetic bacteria, in the rhizosphere, or in the environment, and it was very interesting that there was barely any diurnal change. This could mean that fungi have stronger diurnal patterns of activity in the rhizosphere, or alternatively a large shift was not detected in bacteria due to the selection of eukaryote mRNA.

Chapter 6. Final Discussion and Future Work

The major findings from this thesis are discussed below.

6.1 Rhizosphere fungal diversity is rich, and AM fungi have an uncertain role

The work presented here has shown that *M. giganteus* have a rich fungal diversity, and active community, of root fungi. The identification to species level, showed fungi inhabiting the roots had a range of lifestyles, including parasites, symbionts, and commensals. Roots were dominated by Ascomycota and Basidiomycota, and AMF appeared to have a smaller role than expected. The collation of literature and addition of novel mycobiomes in Chapter 2, lead to the identification of common fungal groups in herbaceous roots, of which Ascomycota classes Sordariomycetes, Leotiomycetes, Dothideomycetes, and Eurotiomycetes, were frequently dominant and abundant, and the realisation that the Basidiomycota were more abundant than the Glomeromycota, across Poaceae hosts and in *M. giganteus* (**Figure 2.4**). Chapter 3 supported these findings, as *M. giganteus* roots were dominated by Ascomycota, with 137 total fungal taxa, compared to ~10 Glomeromycota taxa. These findings were again supported in the meta-transcriptome sequencing of *M. giganteus* roots in Chapter 5, whereby Ascomycota and Basidiomycota taxa dominated microbial eukaryote mRNA transcription, and there was a very small amount of Glomeromycota DNA and annotated mRNA.

However, Glomeromycota dominated ITS RNA samples and over 700,000 reads were aligned to the *R. irregularis* genome in the meta-transcriptome (Chapter 5), which is difficult to explain. However, when Glomeromycota specific nutrient transporter genes were searched for in the meta-transcriptome, very few reads matched, which means they may be on the roots but not as functionally important as once thought. The low abundance of Glomeromycota ribosomal DNA (Chapter 2, 3, and 5), and mRNA (Chapter 5), helps to explain why there was no growth benefit to *M. giganteus*, when an AMF inoculation was applied (Chapter 4), as Glomeromycota do not seem to have a major interaction with this host. Instead, the total diversity of root fungi were vital for ~1/3 of *M.*

giganteus growth, Mg, and Ca nutrition. The positive effect may have come from indirect benefits from saprophytes releasing nutrients from organic matter, or other symbionts, active in the roots. For example, Basidiomycota taxa were found in sequencing of rDNA, rRNA, and mRNA (Chapter 5), and Basidiomycota often form ectomycorrhizal mutualistic interactions with woody and tree species (Allen, *et al.*, 2003).

6.2 Rhizosphere fungal communities are dynamic over time and are influenced by environmental factors

The work presented in this thesis has shown that rhizosphere fungal communities have highly dynamic temporal patterns, from morning to evening (Chapter 5), to seasons and years (Chapter 3). In addition, core and satellite, total fungal and AMF community structures in Chapter 3, and plant root transcription in Chapter 5, were strongly influenced by environmental factors. Throughout this work, it is clear that fungal communities were influenced by time and environmental factors. These two factors are inextricably linked, as the environment conditions are always changing, even in the rhizosphere.

6.2.1 Daily plant-microbe interactions

From day to night there are drastic changes in abiotic factors such as temperature and light, which in turn affect biotic factors, including the build up of newly fixed sugars in the roots over the day, which influences how much sugar is allocated to various functions (Blasing, *et al.*, 2005), including transcription of proteins for plant defenses against the cold and pathogens, and nutrient uptake, in the evening (Chapter 5). The changes in plant daily rhythms, are also influenced by circadian regulation (Farre, 2012), which is likely to influence the activity of fungal species. For example plant defense against pathogens is circadian regulated, which were more active in leaves in the day (McClung, 2011; Wang, *et al.*, 2011b). However, Chapter 5 revealed that plant root defenses were upregulated in the evening, which either had a suppressive effect on the potentially pathogenic *Ophiostphaerella spp.* (Chapter 5), or the fungus may have adapted to be more active in the morning, as fungi have circadian clock regulation (Baker, *et al.*, 2012), which may be influenced by plant defense genes. Circadian regulation and environmental conditions, also influence the

amount and time of carbon allocation to the roots (James, *et al.*, 2008), which was shown in Chapter 5 to affect symbionts, for example the Basidiomycota, *Coniophora puteana*, and Glomeromycota, *Paraglomus laccatum*, were more active in the evening (Chapter 5), possibly as there was more sugar in the roots in the evening, and arbuscular mycorrhizal fungi avoid triggering plant defenses (Tisserant, *et al.*, 2013), so perhaps they can function in the evening better than pathogens. The diurnal activity of rhizosphere microbes are poorly understood, particularly in the Glomeromycota, so these diurnal interactions were a novel discovery.

6.2.2 Seasonal plant-microbe interactions

Seasonal changes in plant growth and development are also regulated by the environment and circadian gene regulation, which may explain the changes in fungal community structure and changing influence of different environmental factors over time in Chapter 3. Over seasons there are changes in abiotic factors including day length, rainfall, temperature, sunlight, drought, floods, frost, storms, snow, nutrient availability etc., which can directly affect fungal communities, by drying out or freezing for example (Trappe and Claridge, 2010) and indirectly, by affecting the plant carbon fixation and stress responses, which will in turn affect carbon allocation and stress responses in the rhizosphere (Graf, *et al.*, 2010).

For example, if there is a drought, as there was between March 2010 - March 2012 in Southwest England (**Figure 6.1**), the plant may produce a drought stress response to become more water efficient. One drought stress mechanism is to reduce stomatal conductance (Hetherington and Woodward, 2003), thereby reducing carbon dioxide entering leaves, which reduces carbon fixation, and sugar allocation to the rhizosphere. Aquaporins and general stress responses are also upregulated in drought (Chaumont and Tyerman, 2014), which could affect carbon allocation to other processes such as leaf growth, again affecting carbon fixation potential, and sugar allocation to the rhizosphere. On the other hand, drought may promote the association with mycorrhizal fungi, which can enhance water uptake for the plant (Smith and Read, 2008).

There is also circadian clock regulation over seasons, which is continually trained by external environmental stimuli, such as day length, using blue light

receptors, and temperature. For example the circadian clock genes Flowering Locus C (Michaels and Amasino, 1999) and Early Flowering Locus (ELF3) (Covington, *et al.*, 2001), time the flowering of plants after certain periods of temperature and light, to optimize flowering to specific times of year, which varies between plant species. Flowering time could affect the nutrients and water needed by the plant, to produce secondary metabolites, such as perfumes to attract bees, and then nutrients to produce healthy seeds. Mycorrhizal fungi have been shown to affect visitation of insect pollinators to plants (Gange and Smith, 2005), and enhance nutrition of plants (Hoeksema, *et al.*, 2010).

There are other plant seasonal dynamics, such as during the plant growing season May - September, *M. giganteus* take up more nutrients from the soil, and exudes more carbon into the soil, than in the autumn, when dieback occurs in the phyllosphere, and nutrients and carbon are stored in the rhizomes (Price, *et al.*, 2004). These plant physiological, and other environmental, temporal changes, will affect the environment in the rhizosphere, such as nutrient availability and plant interactions with different microbes across the year, as was found in Chapter 3.

6.2.3 Longer term plant-microbe interactions

The fungal profiles also changed markedly over the years, the diversity of taxa significantly shifted between 2010 – 2011 (Chapter 3), and the proportions of fungal phyla changed massively between 2011 (Chapter 2 and 3) and 2012 (Chapter 5). In particular, the abundance of the Zygomycota group *Mortierella* dropped from 17.1 % - 23.4 % - 0.1 %, and the Basidiomycota increased from 1.0 % - 2.0 % - 8.1 %, from sampling in October 2010, June 2011 (Chapter 2), to sampling in late August 2012 (Chapter 5), respectively. The massive shift in fungal proportions was likely due to the extreme weather conditions across this time period, when a two year drought in England, from March 2010 - March 2012, was followed by heavy rain and flooding in April - July of 2012. The flooding was likely to change the whole microbiota in the rhizosphere, for example, certain ectomycorrhizal fungi are more tolerant to flooding than others (Stenstrom, 1991). Chris Barnes, a colleague studying the adjacent bioenergy Willow field, also found major shifts in fungal communities from 2011 to 2012.

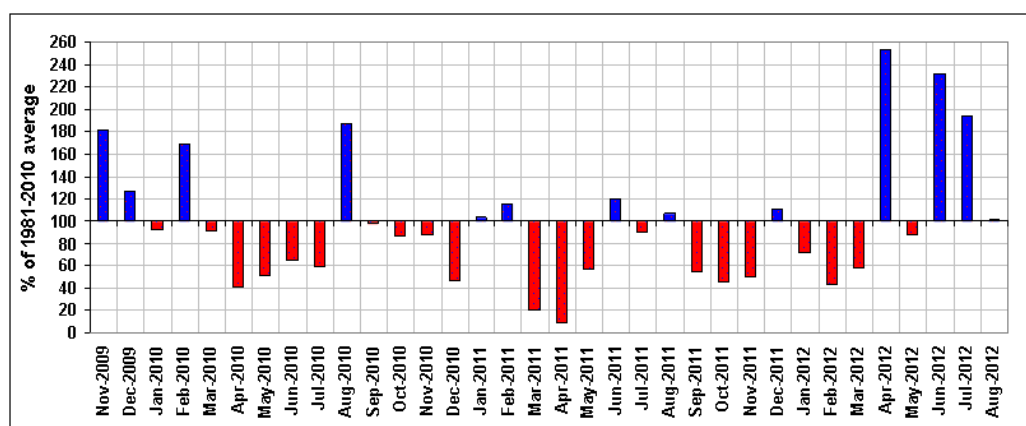


Figure 6.1. MET office statistics for monthly rainfall, expressed as percentage of the 1981-2010 average, for Lowland England from November 2009 to Aug 2012, showing the extreme variation in rainfall, from the drought in March 2010 – March 2012, to the heavy rainfall and floods in April – July 2012 (<http://www.metoffice.gov.uk/climate/uk/interesting/2012-drought> accessed 30.5.14).

6.3 Wider implications and possible future work

There are a number of areas, which need to be researched further, to gain a better understanding of plant-microbe interactions in the rhizosphere, which could have major implications to improve crop growth. Chapter 2 drew attention to the gap in knowledge of general root mycobiomes. There is a need to sequence from a wider range of herbaceous hosts, to develop our understanding of what balance of fungi makes a healthy root mycobiome, compared to unbalanced and diseased mycobiomes, similar to research into core gut microbiomes for human health (Turnbaugh, *et al.*, 2009). In addition to what is living on roots, timing of interactions seems to be a very important issue for plant health.

Root fungi appeared to have circadian rhythms (Chapter 5), and understanding their timings could have major implications for crop health. For example, to deter pathogens, plant defenses could be upregulated, or pesticides applied to soil, at certain times of day. In addition, to promote symbiotic interactions, symbiotic pathways could be enhanced, or fungicide application avoided, at certain times. This work could be developed by analyzing meta-transcriptomes of pathogens and symbionts on roots, over more days, with more sampling times, in a number of important crops.

Fungal communities were also seasonally dynamic (Chapter 3). Plant processes are known to vary seasonally, and be controlled by environment and epigenetic regulation, such as the vernalisation (a prolonged period of cold) of Flowering Locus C is required for flowering, to maximise flowering at the certain times of year (Dennis and Peacock, 2007). Research is being carried out into how flowering time could be optimised to improve yields, for example wheat yields could be enhanced if flowering time is it at maximum solar radiation, as seed filling depends on newly fixed carbon (Snape, *et al.*, 2001).

Environmental conditions also train the plant seasonal clock, which influences when plants begin to grow, flower, die back, and many other things, to optimize biological processes at certain times. Rhizosphere microbes may also have similar mechanisms to use external queues for seasonal change, or be influenced by the changes going on in the plant. Understanding these timings and the influence of environmental conditions, gives us the potential to enhance rhizosphere function at optimal times, including nutrient cycling and defense, while saving energy for other processes including growth.

For example, it is well known certain fungi in the phyllosphere, such as the Basidiomycota Yellow Rust fungus (Wiik and Ewaldz, 2009), are more active after mild winters, and therefore it would be optimal to enhance plant defenses against fungal pathogens after mild winters, when there is no die-off. Understanding when root pathogens are most active, or vulnerable, could enhance defense against them, with genetic engineering to upregulate defense genes after certain weather conditions, or the optimal timing of pesticide use.

Symbiotic interactions could equally be enhanced, by understanding timings of highest infection rates and nutrient cycling activity, plant symbiotic pathways could be upregulated on a seasonal regulation pathway, or after certain environmental conditions, such as nutrient stress. This could be achieved by analyzing the meta-transcriptomes of root mycobiomes over longer periods in the field, which could then be linked to environmental factors, to predict the factors which are most likely to activate different fungal groups, and therefore which pathways to upregulate in response to environmental conditions.

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Appendix

Appendix Table 4.1 The effect of pesticides on AMF colonisation and plant host response. Where the following represent: RD (recommended dose), ND (no data), NE (no effect), RLC (root length colonisation by AMF structures) EP (applied to established plant, rather than seed application, only for benomyl), and * $P < 0.05$ between control and treatment AMF root length colonisation.

Paper	Plant	Pesticide Name	Effect of Pesticide on Plant Growth	Ctrl RLC %	RLC %	Dose
Ipsilantis, <i>et al.</i> (2012)	<i>Capsicum annum</i>	Azadiracthin	No effect	30.0	40	2.79 mg/kg
Diedhiou, <i>et al.</i> (2004)	<i>Zea Mays</i>	Azoxystrobin	No data	62.0	41 *	50 mg/kg
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Azoxystrobin	No data	85.0	15.8 *	RD
Gill, <i>et al.</i> (2013)	<i>Zea mays Paras</i>	Benomyl	Decreased biomass 45 %	40.0	0.0 *	667 mg/kg
Gill, <i>et al.</i> (2013)	<i>Zea mays JH 3459</i>	Benomyl	Decreased biomass 42 %	36.0	0.0 *	667 mg/kg
Pedersen and Sylvia (1997)	<i>Zea Mays</i> (EP)	Benomyl	No growth effect, decrease P	55.0	35.0 *	66.7 mg/kg
O'Connor, <i>et al.</i> (2002)	<i>Medicago minima</i>	Benomyl	40 % reduction	48.8	0.0 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Carrichtera annua</i>	Benomyl	30 % increase	0.0	0.0	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Salvia verbenaca</i>	Benomyl	No effect	79.5	0.0 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Velleia arguta</i>	Benomyl	75 % biomass reduction	47.5	0.0 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Vittadinia gracilis</i>	Benomyl	90 % biomass reduction	56.0	0.0 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Medicago minima</i> (EP)	Benomyl	80 % biomass reduction	80.8	55 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Carrichtera annua</i> (EP)	Benomyl	50 % biomass increase	0.0	0.0	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Salvia verbenaca</i> (EP)	Benomyl	No effect	80.9	57.1 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Velleia arguta</i> (EP)	Benomyl	70 % growth reduction	75.1	54.4 *	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Erodium crinitum</i> (EP)	Benomyl	No effect	61.3	43.2	53.3 mg/kg
O'Connor <i>et al.</i> (2002)	<i>Trifolium subterraneum</i> (EP)	Benomyl	No data (field study)	75.0	50.0 *	53.3 mg/kg
Callaway, <i>et al.</i> (2004)	<i>Centaurea maculosa</i> (EP)	Benomyl	No effect grown alone, reduced biomass with competition	44.2	4.7 *	50 mg/kg
(Gross, <i>et al.</i> , 2010)	<i>Festuca paniculata</i> (EP)	Benomyl	Biomass reduction	ND	ND	50 mg/kg
Camargo-Ricalde, <i>et al.</i> (2010)	<i>Mimosa adenantheroides</i>	Benomyl	70 % biomass reduction	12.2	0.0 *	44 mg/kg
Camargo-Ricalde <i>et al.</i> (2010)	<i>Mimosa calcicola</i>	Benomyl	No effect	14.1	0.0 *	44 mg/kg
Camargo-Ricalde <i>et al.</i> (2010)	<i>Mimosa lacerata</i>	Benomyl	30 % biomass reduction	12.7	0.5 *	44 mg/kg
Camargo-Ricalde <i>et al.</i> (2010)	<i>Mimosa luisana</i>	Benomyl	No effect	14.5	0.0 *	44 mg/kg
Camargo-Ricalde <i>et al.</i> (2010)	<i>Mimosa polyantha</i>	Benomyl	75 % biomass reduction	8.0	0.0 *	44 mg/kg

Camargo-Ricalde <i>et al.</i> (2010)	<i>Mimosa texana</i>	Benomyl	67 % biomass reduction	1.5	0.9	44 mg/kg
Blanke <i>et al.</i> (2011)	<i>Artemisia vulgaris</i> (EP)	Benomyl	Reduced leaf N	68.0	25.0 *	40 mg/kg
Blanke <i>et al.</i> (2011)	<i>Picris hieracioides</i> (EP)	Benomyl	Reduced leaf N	77.0	32.0 *	40 mg/kg
Blanke <i>et al.</i> (2011)	<i>Poa compressa</i> (EP)	Benomyl	No data	40.0	12.0 *	40 mg/kg
Blanke, <i>et al.</i> (2011)	<i>Bromus japonicus</i> (EP)	Benomyl	No data	77.0	5.0 *	40 mg/kg
Pedersen and Sylvia (1997)	<i>Zea Mays</i> (EP)	Benomyl	Small growth decrease, decrease P	55.0	20.0 *	26.7 mg/kg
Frank, <i>et al.</i> (2003)	<i>Poa pratensis</i>	Benomyl	1/3 growth reduction	13.0	1.4 *	250 mg/kg
Pedersen and Sylvia (1997)	<i>Zea Mays</i> (EP)	Benomyl	Small growth decrease, decrease P	55.0	43.0 *	200 mg/kg
Welsh, <i>et al.</i> (2010)	<i>Spartina patens</i> (EP)	Benomyl	Reduced growth	26.3	11.5 *	200 mg/kg
Zhang, <i>et al.</i> (2011b)	<i>Medicago sativa</i> (EP)	Benomyl	Biomass increase at low plant densities, decrease at high densities	24.0	6.5 *	17.1 mg/kg
Allison, <i>et al.</i> (2007)	<i>Poa compressa</i>	Benomyl	Increased growth, reduced N	17.5	13.0	16.7 mg/kg
Allison, <i>et al.</i> (2007)	<i>Bromus inermis</i>	Benomyl	No effect	15.0	21.0	16.7 mg/kg
Allison <i>et al.</i> (2007)	<i>Potentilla recta</i>	Benomyl	No effect	15.0	15.0	16.7 mg/kg
Allison <i>et al.</i> (2007)	<i>Hieracium caespitosum</i>	Benomyl	Increased growth	15.0	14.0	16.7 mg/kg
Allison <i>et al.</i> (2007)	<i>Plantago lanceolata</i>	Benomyl	No effect	23.0	18.0	16.7 mg/kg
Allison <i>et al.</i> (2007)	<i>Achillea millefolium</i>	Benomyl	Increased growth	18.0	19.0	16.7 mg/kg
Cahill, <i>et al.</i> (2008)	<i>Campanula rotundifolia</i> (EP)	Benomyl	Reduced no. flowering stems	4.8	3.2	16.7 mg/kg
Cahill, <i>et al.</i> (2008)	<i>Cerastium arvense</i> (EP)	Benomyl	Reduced flowering	5.6	3.7	16.7 mg/kg
Cahill, <i>et al.</i> (2008)	<i>Achillea millefolium</i> (EP)	Benomyl	Reduced no. flowering stems	13.2	8.8	16.7 mg/kg
Cahill, <i>et al.</i> (2008)	<i>Aster laevis</i> (EP)	Benomyl	No effect on flowering	15.4	10.3	16.7 mg/kg
Cahill, <i>et al.</i> (2008)	<i>Erigeron philadelphicus</i> (EP)	Benomyl	No effect on flowering	16.8	11.2	16.7 mg/kg
Cahill, <i>et al.</i> (2008)	<i>Solidago missouriensis</i> (EP)	Benomyl	No effect on flowering	31.3	20.9	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	Sedges (EP)	Benomyl	Increased growth, Lowered P, increased N	ND	ND	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	<i>Plantago lanceolata</i> (EP)	Benomyl	Decreased growth, lowered P, no effect N	62.0	23.0 *	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	<i>Galium</i> (EP)	Benomyl	No effect growth, lowered P, no effect N	78.0	15.0 *	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	Perennial Forbs (EP)	Benomyl	Decreased growth, decreased P, no effect N	ND	ND	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	Grasses (EP)	Benomyl	Increased growth, lowered P, increased N	ND	ND	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	<i>Agrostis</i> (EP)	Benomyl	No effect growth, decreased P, increased N	ND	ND	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	Legumes (EP)	Benomyl	Decreased growth, decreased P, no effect N	ND	ND	16.7 mg/kg
Karanika, <i>et al.</i> (2008)	<i>Daricnium</i> (EP)	Benomyl	Increased growth, decreased P, no effect N	ND	ND	16.7 mg/kg

Wilson and Williamson (2008)	<i>Mixed Prairie grass</i> (EP)	Benomyl	Reduced flowering	13.7	4.2 *	16.7 mg/kg
Daleo, <i>et al.</i> (2008)	<i>Spartina densiflora</i>	Benomyl	Increased growth at low nutrient levels, reduced growth at high nutrient levels	48.0	4.6 *	100 mg/kg
Daleo, <i>et al.</i> (2008)	<i>Spartina alterniflora</i>	Benomyl	Increased growth at high and low nutrient levels	0.0	0.0	100 mg/kg
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i> (EP)	Benomyl	No effect	21.5	26.5	100 mg/kg
Zhang, <i>et al.</i> (2011a)	<i>Medicago sativa</i>	Benomyl	1/3 biomass reduction	50.0	10.0 *	100 mg/kg
Welc, <i>et al.</i> (2010)	<i>Cucumis sativus</i>	Benomyl	No effect	95.1	64.2 *	10.0 mg/kg
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i> (EP)	Benomyl	No effect	21.5	21.5	1 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captafol	54 % biomass reduction	52.0	28.0 *	10 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captafol	69 % biomass reduction	52.0	16.0 *	25 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captafol	78 % biomass reduction	52.0	0.0 *	50 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captafol	78 % biomass reduction	52.0	0.0 *	100 mg/kg
Jin, <i>et al.</i> (2013)	<i>Pisum sativum</i>	Captan	No effect biomass	28.0	29.0	RD
Jin, <i>et al.</i> (2013)	<i>Cicer arietinum</i>	Captan	No effect biomass	25.0	26.0	RD
Kangwankraipha isan, <i>et al.</i> (2013)	<i>Lindenbergia philippensis</i>	Captan	Decreased Zn uptake	47.9	10.0 *	80 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captan	33 % biomass reduction	52.0	36.0	10 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captan	52 % biomass reduction	52.0	28.0	25 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captan	59 % biomass reduction	52.0	28.0	50 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Captan	66 % biomass reduction	52.0	20.0 *	100 mg/kg
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Captan	No data	79.0	70.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Captan	No data	79.0	87.0 *	Half RD
Jin, <i>et al.</i> (2013)	<i>Cicer arietinum</i>	Carbathiin Thiabendazole	No biomass effect without AMF, 27 % reduction with AMF	25.0	25.5	RD
Jin, <i>et al.</i> (2013)	<i>Pisum sativum</i>	Carbathiin Thiram	No biomass effect without AMF, 1/3 biomass and P reduction with AMF	28.0	26.0	RD
Jin, <i>et al.</i> (2013)	<i>Cicer arietinum</i>	Carbathiin Thiram	No effect biomass	ND	ND	RD
Dostalek, <i>et al.</i> (2013)	<i>Aster amellus</i>	Carbendazim	Plant composition changed in nutrient poor soil	9.5	4.1	100 ml m ⁻²
Ipsilantis <i>et al.</i> (2012)	<i>Capsicum annum</i>	Carbendazim	No effect	30.0	0 *	2 mg/kg
Schweiger, <i>et al.</i> (2001)	<i>Pisum sativum</i>	Carbendazim	Slight stimulatory effect	66.0	67.0	RD
Schweiger <i>et al.</i> (2001)	<i>Pisum sativum</i>	Carbendazim	Slight stimulatory effect	66.0	27.0	RD x 100
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Carbendazim	No data	79.0	63.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Carbendazim	No data	79.0	77.0	Half RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Ceresan	No data	79.0	62.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Ceresan	No data	79.0	79.0	Half RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i> (EP)	Chinosol	No data	85.0	90.4	RD

Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Chlorothalonil	No data	85.0	50.2 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Ciproconazole	No data	85.0	38.1 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Ciprodinyl + Fludioxonil	No data	85.0	26.4 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Copper oxychloride	No data	85.0	81.4	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Copper oxychloride	No data	79.0	59.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Copper oxychloride	No data	79.0	74.0 *	Half RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Dichlofluanide	No data	85.0	38.4 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Dimetomorph + Folpet	No data	85.0	1.1 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Etridiazole	No data	85.0	30.9 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Fenarimole	No data	85.0	0 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Fenhexamide	No data	85.0	30.4 *	RD
Zocco, <i>et al.</i> (2008)	<i>Daucus carota</i>	Fenhexamide	Massively reduced	34.0	29.0	200 mg l ⁻²
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i>	Fenpropimorph	No effect	21.5	22.0	1.25 mg/kg
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i>	Fenpropimorph	No effect	21.5	28.7	125 mg/kg
Zocco, <i>et al.</i> (2008)	<i>Daucus carota</i>	Fenpropimorph	Massively reduced	34.0	28	200 mg/l
Schweiger <i>et al.</i> (2001)	<i>Pisum sativum</i>	Fenpropimorph + Propiconazole	Marginally reduced	66.0	66.0	RD
Jin, <i>et al.</i> (2013)	<i>Pisum sativum</i>	Fludioxonil Metalaxyl Glycerin	1/3 biomass reduction without AMF, 18 % biomass reduction with AMF	28.0	25.0	RD
Jin, <i>et al.</i> (2013)	<i>Cicer arietinum</i>	Fludioxonil Metalaxyl Glycerin	No effect without AMF, 18 % biomass and 27 % P reduction with AMF	25.0	25.0	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Flutholanil	No data	85.0	80.7	RD
Marin, <i>et al.</i> (2002)	<i>Cardoon</i>	Folpet	Increased growth	No data	No effect	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Fosethyl-aluminium	No data	85.0	50.9 *	RD
Roberts and Anderson (2001)	<i>Solanum lycopersicum</i>	Garlic Mustard leechates	Reduced germination	No data	Reduced	RD
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Gypsum	77 % biomass reduction	52.0	20.0 *	10 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Gypsum	77 % biomass reduction	52.0	10.0 *	25 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Gypsum	80 % biomass reduction	52.0	0.0 *	50 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Gypsum	82 % biomass reduction	52.0	0.0 *	100 mg/kg
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Hymexazole	No data	85.0	50.5 *	RD
Gange and West (1994)	<i>Plantago lanceolata</i>	Iprodione	Reduced biomass	45.0	8.0 *	2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Conyza canadensis</i>	Iprodione	No effect	25.0	25.5	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Gnaphalium uliginosum</i>	Iprodione	No effect	39.0	18 *	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Sonchus oleraceus</i>	Iprodione	No effect	28.0	21.5	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Spergula arvensis</i>	Iprodione	No effect	40.5	30.0 *	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Stellaria media</i>	Iprodione	No effect	14.5	14.5	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Tripleurospermum inodorum</i>	Iprodione	Reduced growth	42.0	31.0 *	3.2 g m ⁻²

Gange, <i>et al.</i> (1990)	<i>Veronica persica</i>	Iprodione	Reduced growth	23.0	14.5 *	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Vicia tetrasperma</i>	Iprodione	Reduced growth	33.5	18.0 *	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Medicago lupulina</i>	Iprodione	Reduced growth	31.0	17.0 *	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Poa annua</i>	Iprodione	No effect	17.0	10.5	3.2 g m ⁻²
Gange, <i>et al.</i> (1990)	<i>Agrostis stolonifera</i>	Iprodione	No effect	29.5	16.5 *	3.2 g m ⁻²
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Iprodione	No data	85.0	0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Iprodione	No data	79.0	59.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Iprodione	No data	79.0	73.0 *	Half RD
Diedhiou, <i>et al.</i> (2004)	<i>Zea Mays</i>	Kresoxim-methyl	No data	62.0	51.0	50 mg/kg
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Kresoxim-methyl	No data	85.0	9.3 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Mancozeb	No data	85.0	0 *	RD
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Mancozeb	48 % biomass reduction	52.0	32.0	10 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Mancozeb	55 % biomass reduction	52.0	28.0	25 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Mancozeb	60 % biomass reduction	52.0	24.0 *	50 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Mancozeb	69 % biomass reduction	52.0	0.0 *	100 mg/kg
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Mancozeb	No data	79.0	69.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Mancozeb	No data	79.0	79.0	Half RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Mepanipyrim	No data	85.0	12.4 *	RD
Jin, <i>et al.</i> (2013)	<i>Pisum sativum</i>	Metalaxyl	1/3 biomass reduction without AMF, 22 % biomass reduction with AMF	28.0	22.5 *	RD
Jin, <i>et al.</i> (2013)	<i>Cicer arietinum</i>	Metalaxyl	No effect without AMF, 30 % reduction biomass and P with AMF	25.0	24.0	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Methalaxyl + Mancozeb	No data	85.0	50.7 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Methalaxyl = Mephenoxam	No data	85.0	38.3 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Miclobutanil	No data	85.0	4.3 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Penconazole	No data	85.0	14 *	RD
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Pentachloronitr obenzene	74 % biomass reduction	52.0	12.0 *	10 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Pentachloronitr obenzene	78 % biomass reduction	52.0	4.0 *	25 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Pentachloronitr obenzene	82 % biomass reduction	52.0	0.0 *	50 mg/kg
Parvathi, <i>et al.</i> (1985)	<i>Arachis hypogaea</i>	Pentachloronitr obenzene	84 % biomass reduction	52.0	0.0 *	100 mg/kg
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Prochloraz	No data	85.0	10.4 *	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Propamocarb	No data	85.0	90.4	RD
Marin, <i>et al.</i> (2002)	<i>Cardoon</i>	Propamocarb	Increased growth	No data	NE	RD
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i>	Propiconazole	No effect	21.5	19.5	0.21 mg/kg
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i>	Propiconazole (Tilt Top)	No effect	21.5	26.9	0.83 mg/kg
Kjoller and Rosendahl (2000)	<i>Cucumis sativus</i>	Propiconazole (Tilt Top)	No effect	21.5	24.3	83 mg/kg

Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Quintozene	No data	79.0	61.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Quintozene	No data	79.0	80.0	Half RD
Ipsilantis <i>et al.</i> (2012)	<i>Capsicum annum</i>	Spinosad	No effect	30.0	25.0	2 mg/kg
Ipsilantis <i>et al.</i> (2012)	<i>Capsicum annum</i>	Terpenes	Increased growth at 40 days	30.0	30.0	7 mg/kg
Pattinson, <i>et al.</i> (1997)	<i>Gossypium hirsutum</i>	Terraclor	No effect of biomass or P	53.7	54.5	RD
Pattinson <i>et al.</i> (1997)	<i>Gossypium hirsutum</i>	Terrazole	No effect of biomass or P	53.7	52.6	RD
Hernandez-Dorrego (2010)	<i>Allium porrum</i>	Tetramethylthiuram disulfide	No data	85.0	2.7 *	RD
Jin, <i>et al.</i> (2013)	<i>Pisum sativum</i>	Thiram	No effect biomass	28.0	28.0	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Thiram	No data	79.0	67.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Thiram	No data	79.0	81.0	Half RD
Wilson and Williamson (2008)	<i>Pascopyron smithii</i>	Thiophanate methyl (Topsin-M)	No effect	17.5	2.0 *	50 mg/kg
Wilson and Williamson (2008)	<i>Andropogon gerardii</i>	Thiophanate methyl (Topsin-M)	Significant biomass reduction	24.5	7.7 *	50 mg/kg
Jin, <i>et al.</i> (2013)	<i>Cicer arietinum</i>	Trifloxystrobin, Metalaxyl	20 % biomass reduction without AMF, 22 % biomass and 1/3 P reduction with AMF	25.0	25.0	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Zineb	No data	79.0	60.0 *	RD
Sreenivasa and Bagyaraj (1989)	<i>Chloris gayana</i>	Zineb	No data	79.0	72.0 *	Half RD

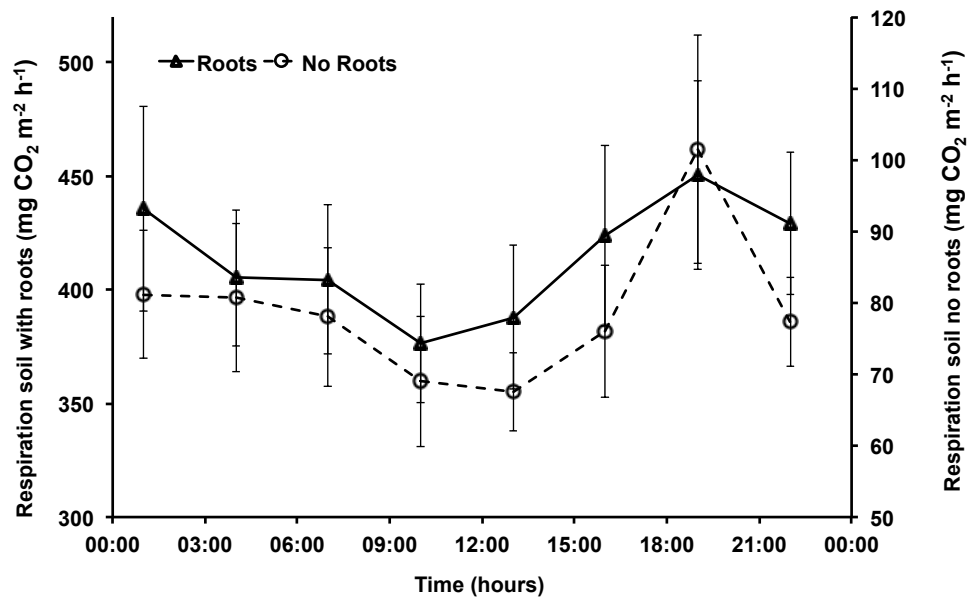
Appendix Table 4.2 Analysis of Variance (ANOVA) statistics of dry weight (DW) and fresh weights (FW), of rhizomes, shoots, and rhizomes biomass, testing for variance between treatments, correcting for row and column variability, for **Experiment 1**. a) raw data, excluding treatment 15 (mg/kg) from analysis, b) log-transformed data, excluding treatment 15 (mg/kg). Where d.f. = degrees of freedom, v. r. = variance ratio, F. pr = Test statistic, * = $p < 5\%$.

(a)

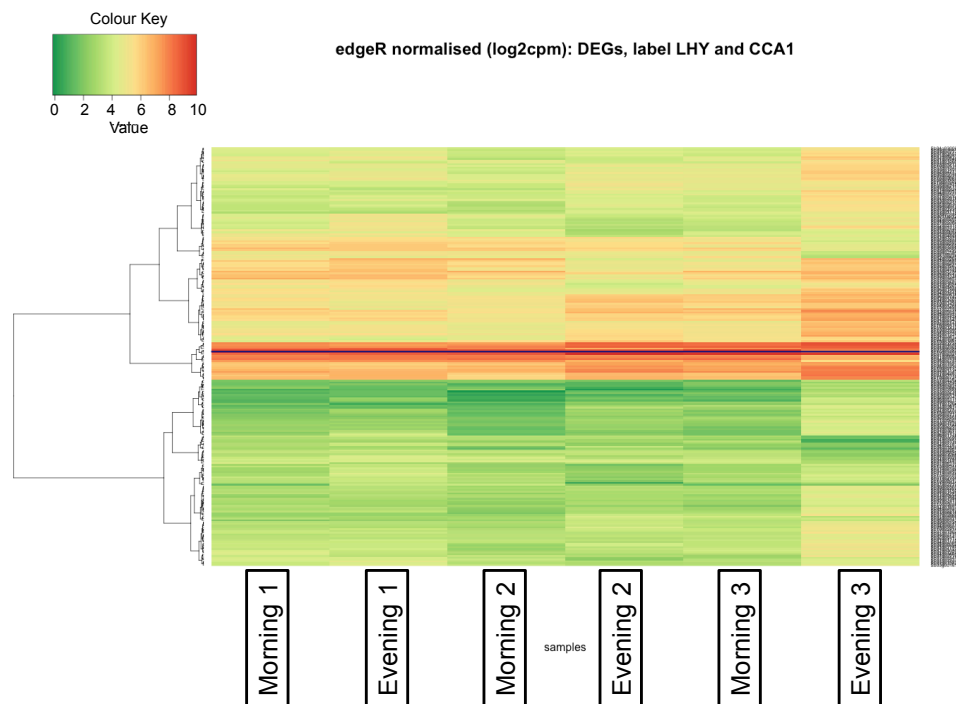
Variable	Analysis of variance		d.f.	v.r.	F pr.
DW_Rhizome	Column.row stratum	Treatment	6	0.68	0.667
		Residual	35	1.56	
DW_Roots	Column.row stratum	Treatment	6	1.11	0.377
		Residual	35	1.71	
DW_Shoot	Column.row stratum	Treatment	6	0.38	0.889
		Residual	35	2.61	
FW_Rhizome	Column.row stratum	Treatment	6	0.95	0.47
		Residual	35	1.19	
FW_Roots	Column.row stratum	Treatment	6	1.51	0.202
		Residual	35	1.6	

(b)

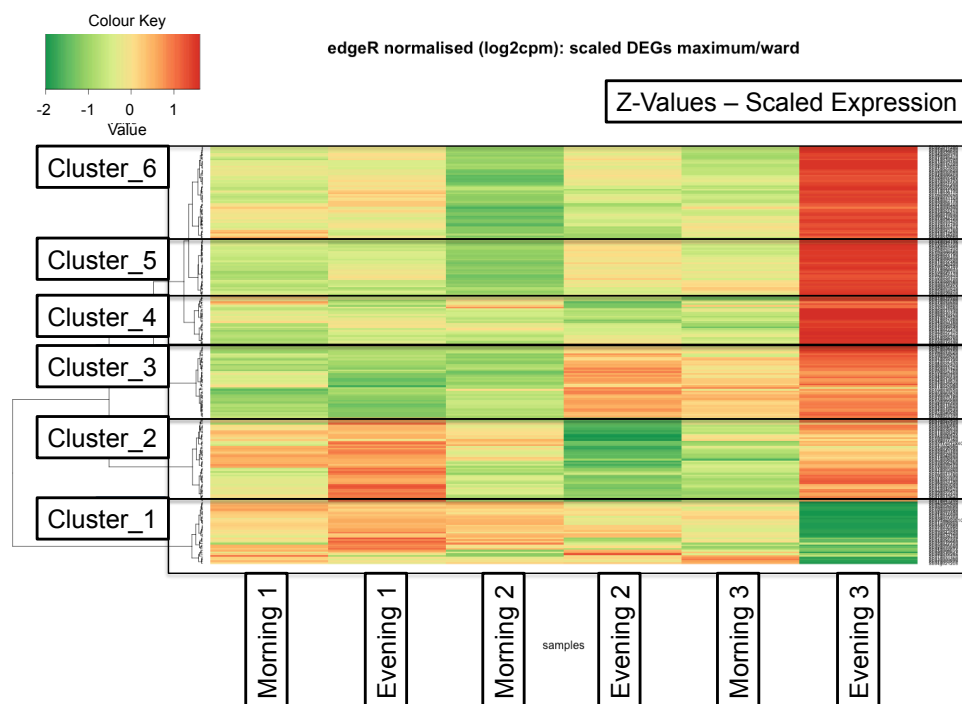
Variable	Analysis of variance		d.f.	v.r.	F pr.
Logged data					
DW_Rhizome	Column.row stratum	Treatment	6	0.91	0.499
		Residual	35	1.46	
DW_Roots	Column.row stratum	Treatment	6	0.58	0.746
		Residual	35	1.61	
DW_Shoot	Column.row stratum	Treatment	6	0.56	0.763
		Residual	35	1.9	
FW_Rhizome	Column.row stratum	Treatment	6	1.05	0.409
		Residual	35	1.14	
FW_Roots	Column.row stratum	Treatment	6	0.68	0.667
		Residual	35	1.63	



Appendix Figure 5.1. Mean (\pm StDev) respiration, taken every three hours, from five replicate plots, in a commercial *Miscanthus* field, with 'Roots', or 'No roots' (root exclusion plot), from 1st August to 10th September 2011.



Appendix Figure 5.2. Gene expression heatmap of all edgeR normalised DEGs, over three consecutive days. Replicates of morning and evening are merged. Gene identifiers are in small text on the right of expression profiles.



Appendix Figure 5.3. Scaled gene expression heatmap of DEGs using Z-values, over three consecutive days. Replicates of morning and evening are merged, and clusters shown within boxes. Gene identifiers are in small text on the right of expression profiles.

Appendix Table 5.1. Differentially expressed genes (DEGs) between morning and evening overlap between days. Hit names and definitions, annotated using the *Arabidopsis thaliana* (*At*) genome provided by The Arabidopsis Information Resource (TAIR) database, or the *Sorghum bicolor* (*Sb*) genome, only when an *A. thaliana* hit or definition was not available. DEGs were clustered into five groups, based on hierarchical clustering of gene expression profiles.

Cluster 1

<i>At</i> or <i>Sb</i> hit name	<i>At</i> or <i>Sb</i> hit define
Sb04g021205	similar to Putative uncharacterized protein P0461D06.29
AT1G01060.1	Homeodomain-like superfamily protein
AT1G20160.1	Subtilisin-like serine endopeptidase family protein
AT3G18830.1	polyol/monosaccharide transporter 5
AT1G64940.1	cytochrome P450, family 87, subfamily A, polypeptide 6
AT5G33406.1	hAT dimerisation domain-containing protein / transposase-related
AT3G24870.1	Helicase/SANT-associated, DNA binding protein
AT3G26740.1	CCR-like
AT5G64530.1	xylem NAC domain 1
AT4G27450.1	Aluminium induced protein with YGL and LRDR motifs
AT4G29190.1	Zinc finger C-x8-C-x5-C-x3-H type family protein
AT4G13420.1	high affinity K ⁺ transporter 5
AT2G03530.2	ureide permease 2
AT4G11450.1	Protein of unknown function (DUF3527)

AT3G30530.1	basic leucine-zipper 42
AT2G38470.1	WRKY DNA-binding protein 33
AT4G35780.1	ACT-like protein tyrosine kinase family protein
AT5G13290.2	Protein kinase superfamily protein
AT1G08320.1	bZIP transcription factor family protein
AT3G13340.2	Transducin/WD40 repeat-like superfamily protein
AT1G51950.1	indole-3-acetic acid inducible 18
AT4G32110.1	Beta-1,3-N-Acetylglucosaminyltransferase family protein
AT1G09570.1	phytochrome A
AT1G09570.1	phytochrome A
AT1G09570.1	phytochrome A
AT4G33000.2	calcineurin B-like protein 10
AT5G59780.2	myb domain protein 59
AT1G48410.3	Stabilizer of iron transporter SufD / Polynucleotidyl transferase
AT1G09900.1	Pentatricopeptide repeat (PPR-like) superfamily protein
AT5G58380.1	SOS3-interacting protein 1
AT1G51200.2	A20/AN1-like zinc finger family protein
AT5G21170.1	5'-AMP-activated protein kinase beta-2 subunit protein
AT1G66330.2	senescence-associated family protein
AT3G17510.1	CBL-interacting protein kinase 1
AT1G78020.1	Protein of unknown function (DUF581)

Cluster 2

At or Sb hit name	At or Sb hit define
AT4G38660.1	Pathogenesis-related thaumatin superfamily protein
AT5G67210.1	Protein of unknown function (DUF579)
AT2G06925.1	Phospholipase A2 family protein
Sb05g016910	similar to Putative uncharacterized protein
Sb02g023740	similar to Putative uncharacterized protein P0027G10.62
AT2G28690.1	Protein of unknown function (DUF1635)
AT5G39670.1	Calcium-binding EF-hand family protein
AT3G22410.1	Sec14p-like phosphatidylinositol transfer family protein
AT3G05800.1	AtBS1(activation-tagged BRI1 suppressor 1)-interacting factor 1
AT2G28660.1	Chloroplast-targeted copper chaperone protein
Sb06g021400	similar to H0306B06.4 protein
Sb04g033190	similar to Pherophorin-C2 protein precursor
AT5G54500.1	flavodoxin-like quinone reductase 1
AT1G75500.2	Walls Are Thin 1
AT1G63100.1	GRAS family transcription factor
AT1G67340.1	HCP-like superfamily protein with MYND-type zinc finger
AT5G47230.1	ethylene responsive element binding factor 5
AT4G13180.1	NAD(P)-binding Rossmann-fold superfamily protein
AT1G71870.1	MATE efflux family protein
AT3G29635.1	HXXXD-type acyl-transferase family protein
AT2G29130.1	laccase 2
AT5G03170.1	FASCICLIN-like arabinogalactan-protein 11
AT5G60020.1	laccase 17
AT3G48280.1	cytochrome P450, family 71, subfamily A, polypeptide 25
AT1G65680.1	expansin B2
AT3G48950.1	Pectin lyase-like superfamily protein

AT1G66810.1	Zinc finger C-x8-C-x5-C-x3-H type family protein
AT5G46900.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT1G21460.1	Nodulin MtN3 family protein
AT4G18260.1	Cytochrome b561/ferric reductase transmembrane protein family
AT5G48485.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT2G45180.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT5G13140.1	Pollen Ole e 1 allergen and extensin family protein
AT3G62760.1	Glutathione S-transferase family protein
AT3G18000.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT5G37660.2	plasmodesmata-located protein 7
AT2G38320.1	TRICHOME BIREFRINGENCE-LIKE 34
Sb01g050435	weakly similar to Expansin-B7 precursor
AT1G65680.1	expansin B2
AT1G65680.1	expansin B2
AT5G60210.2	ROP interactive partner 5
AT5G54500.1	flavodoxin-like quinone reductase 1
AT5G05390.1	laccase 12
AT1G03010.1	Phototropic-responsive NPH3 family protein
AT4G34500.1	Protein kinase superfamily protein
Sb03g002930	similar to Putative uncharacterized protein
AT3G08030.1	Protein of unknown function, DUF642

Cluster 3

At or Sb hit name	At or Sb hit define
AT3G58000.1	VQ motif-containing protein
AT1G76690.1	12-oxophytodienoate reductase 2
AT2G46950.1	cytochrome P450, family 709, subfamily B, polypeptide 2
AT3G05950.1	RmlC-like cupins superfamily protein
AT5G39160.1	RmlC-like cupins superfamily protein
AT3G54020.1	Arabidopsis Inositol phosphorylceramide synthase 1
AT2G46950.1	cytochrome P450, family 709, subfamily B, polypeptide 2
AT5G53050.3	alpha/beta-Hydrolases superfamily protein
AT2G37010.1	non-intrinsic ABC protein 12
AT5G58160.1	actin binding
AT3G01120.1	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein
AT1G10360.1	glutathione S-transferase TAU 18
AT4G33050.3	calmodulin-binding family protein
AT4G08250.1	GRAS family transcription factor
AT2G26560.1	phospholipase A 2A
AT5G55050.1	GDSL-like Lipase/Acylhydrolase superfamily protein
AT4G16730.1	terpene synthase 02
AT4G02780.1	Terpenoid cyclases/Protein prenyltransferases superfamily protein
Sb01g032040	similar to Putative uncharacterized protein
AT3G53450.1	Putative lysine decarboxylase family protein
AT3G05950.1	RmlC-like cupins superfamily protein
Sb03g013810	similar to Putative uncharacterized protein
Sb04g026690	Predicted protein

AT5G06320.1	NDR1/HIN1-like 3
AT5G59880.1	actin depolymerizing factor 3
Sb10g024250	similar to Putative uncharacterized protein
AT1G07160.1	Protein phosphatase 2C family protein
AT3G01650.1	RING domain ligase1
AT1G79460.1	Terpenoid cyclases/Protein prenyltransferases superfamily protein
AT3G22360.1	alternative oxidase 1B
AT3G54420.1	homolog of carrot EP3-3 chitinase
AT2G44260.1	Plant protein of unknown function (DUF946)
AT5G23960.2	terpene synthase 21
AT4G15560.1	Deoxyxylulose-5-phosphate synthase
AT1G64380.1	Integrase-type DNA-binding superfamily protein
AT5G20250.4	Raffinose synthase family protein
Sb09g019020	similar to Putative uncharacterized protein OJ1354_D07.15
AT4G39830.1	Cupredoxin superfamily protein
AT3G11480.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT2G17220.1	Protein kinase superfamily protein

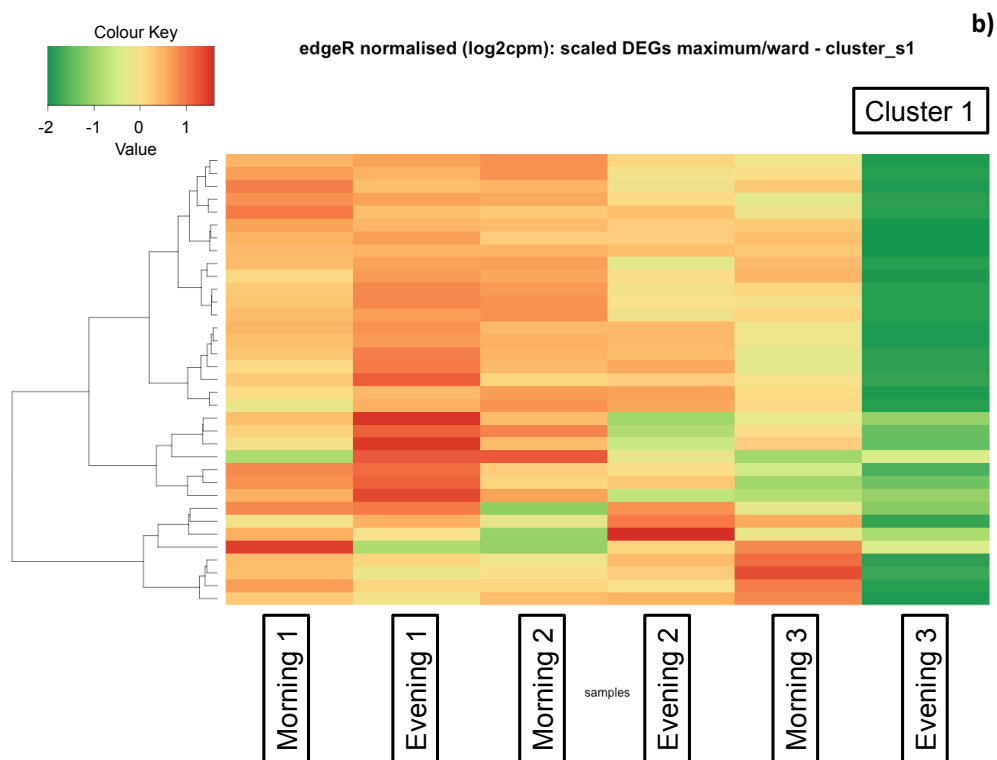
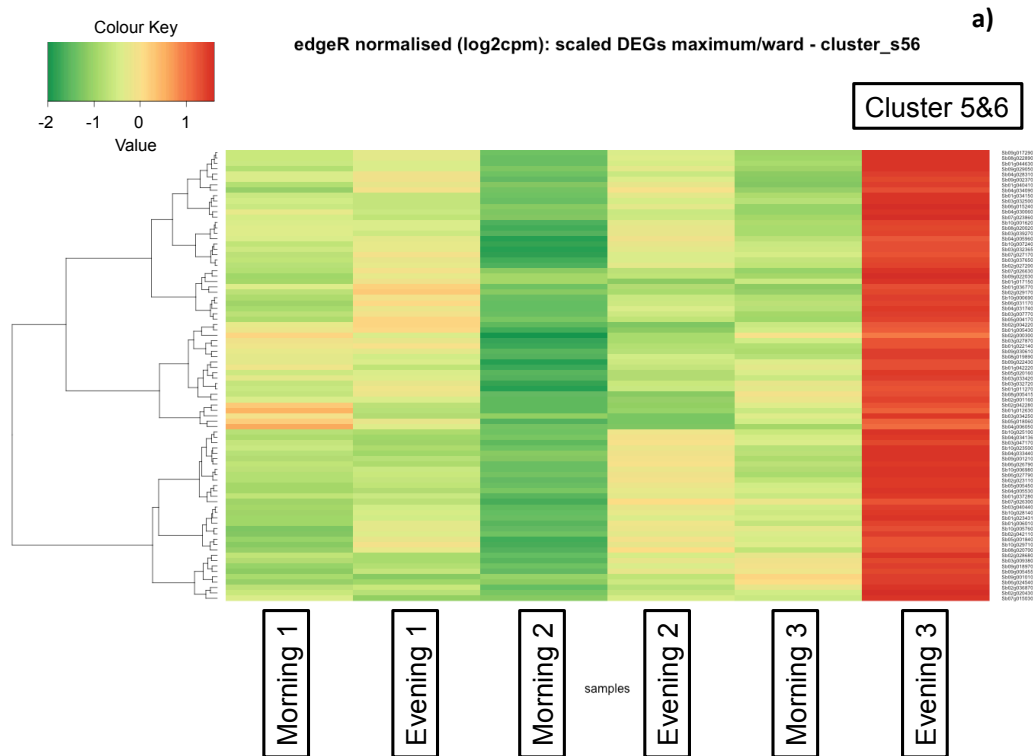
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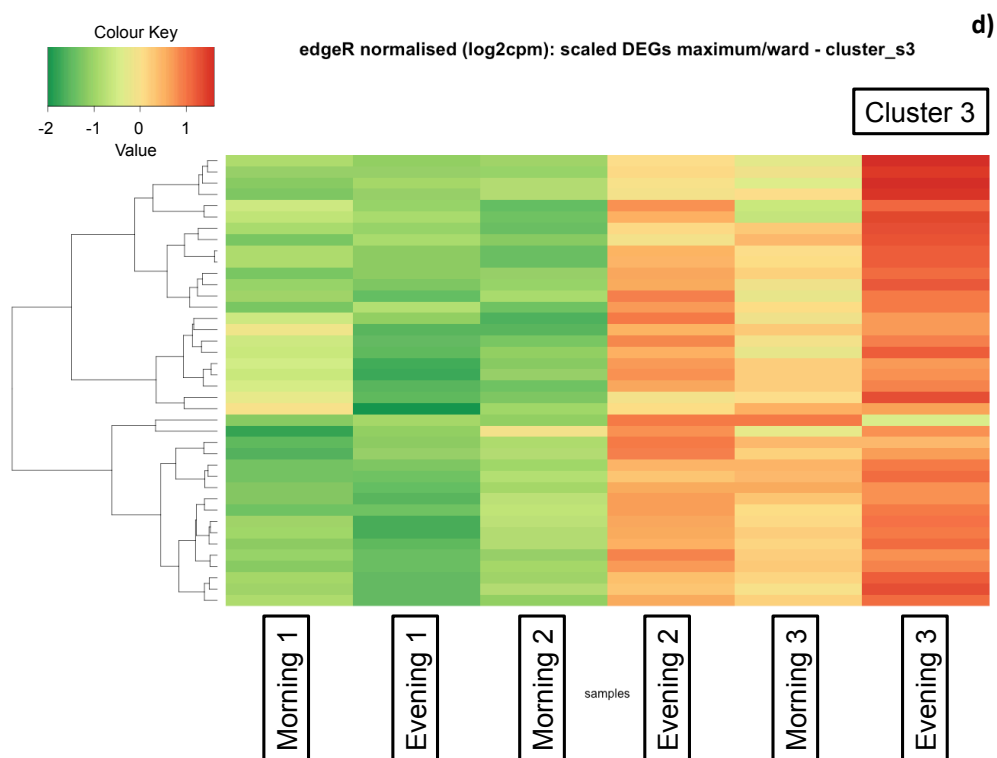
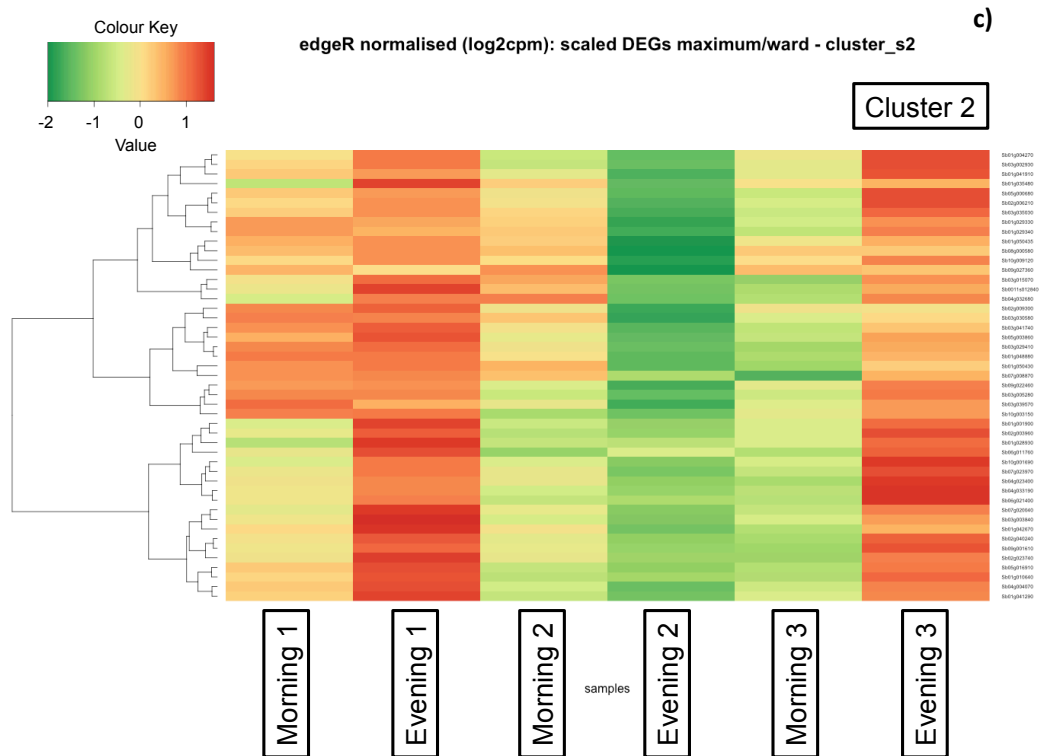
<i>At</i> or <i>Sb</i> hit name	<i>At</i> or <i>Sb</i> hit define
AT4G14540.1	nuclear factor Y, subunit B3
AT2G46950.1	cytochrome P450, family 709, subfamily B, polypeptide 2
Sb10g012190	similar to Putative uncharacterized protein
AT2G32300.1	uclacyanin 1
Sb04g029780	similar to Putative uncharacterized protein
Sb07g022350	weakly similar to Putative uncharacterized protein
AT3G20060.1	ubiquitin-conjugating enzyme19
AT2G02040.1	peptide transporter 2
AT5G58400.1	Peroxidase superfamily protein
Sb02g027270	similar to Putative uncharacterized protein
AT1G78230.1	Outer arm dynein light chain 1 protein
AT1G68840.1	related to ABI3/VP1 2
Sb03g029400	similar to Putative uncharacterized protein P0707D10.15
AT2G32300.1	uclacyanin 1
AT3G48410.1	alpha/beta-Hydrolases superfamily protein
AT2G40330.1	PYR1-like 6
AT2G01770.1	vacuolar iron transporter 1
AT2G14880.1	SWIB/MDM2 domain superfamily protein
AT5G64570.1	beta-D-xylosidase 4
AT2G18890.1	Protein kinase superfamily protein
AT5G57540.1	xyloglucan endotransglucosylase/hydrolase 13
AT5G42800.1	dihydroflavonol 4-reductase
AT1G22360.1	UDP-glucosyl transferase 85A2
AT5G42510.1	Disease resistance-responsive (dirigent-like protein) family protein
AT2G07050.1	cycloartenol synthase 1
AT2G19590.1	ACC oxidase 1
AT3G45140.1	lipoxygenase 2
AT4G13420.1	high affinity K ⁺ transporter 5

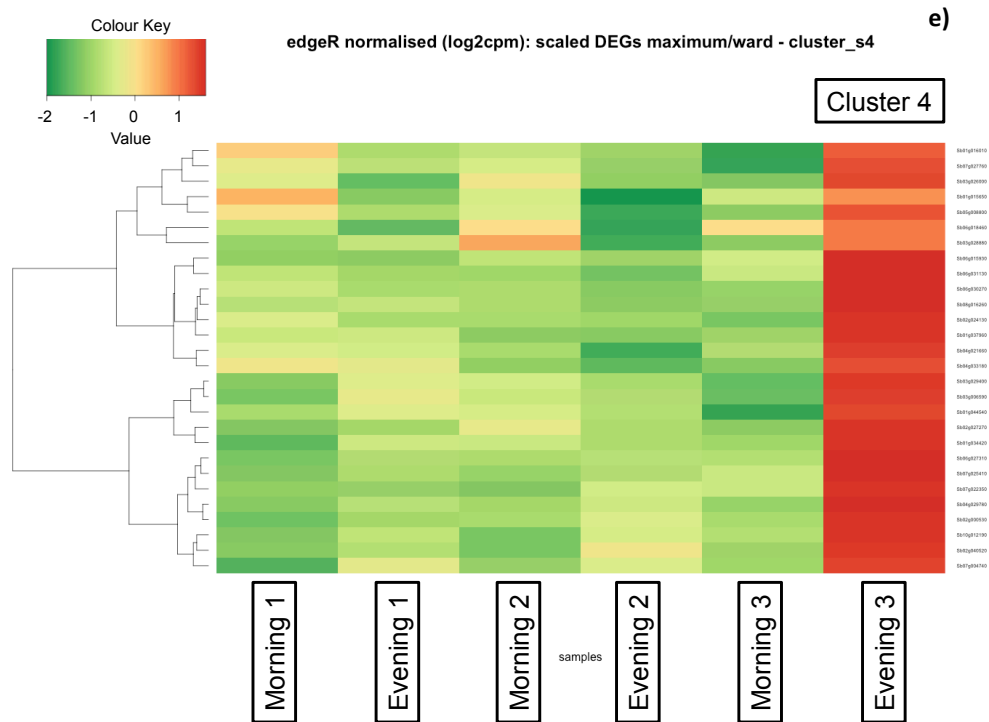
Cluster 5&6

At or Sb hit name	At or Sb hit define
AT2G43120.1	RmlC-like cupins superfamily protein
AT2G04400.1	Aldolase-type TIM barrel family protein
AT5G26330.1	Cupredoxin superfamily protein
AT5G11590.1	Integrase-type DNA-binding superfamily protein
AT2G38870.1	Serine protease inhibitor, potato inhibitor I-type family protein
AT4G13395.1	ROTUNDIFOLIA like 12
AT2G38750.1	annexin 4
AT4G36810.1	geranylgeranyl pyrophosphate synthase 1
Sb02g028680	weakly similar to Os09g0493000 protein
Sb08g020700	similar to Putative uncharacterized protein
AT1G31350.1	KAR-UP F-box 1
AT2G27080.2	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
Sb02g042110	weakly similar to Putative uncharacterized protein
AT5G48930.1	hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl transferase
AT1G53680.1	glutathione S-transferase TAU 28
AT3G43440.1	jasmonate-zim-domain protein 11
AT1G44350.1	IAA-leucine resistant (ILR)-like gene 6
AT2G27690.1	cytochrome P450, family 94, subfamily C, polypeptide 1
AT5G50380.1	exocyst subunit exo70 family protein F1
AT5G49480.1	Ca ²⁺ -binding protein 1
AT4G31805.1	WRKY family transcription factor
AT1G77450.1	NAC domain containing protein 32
AT2G26110.1	Protein of unknown function (DUF761)
Sb06g027790	similar to OSIGBa0113113.1 protein
AT1G21910.1	Integrase-type DNA-binding superfamily protein
Sb06g026790	similar to Putative uncharacterized protein
AT3G12500.1	basic chitinase
AT1G75540.1	salt tolerance homolog2
AT5G16990.1	Zinc-binding dehydrogenase family protein
AT5G51990.1	C-repeat-binding factor 4
Sb04g034136	similar to Expressed protein
AT1G13110.1	cytochrome P450, family 71 subfamily B, polypeptide 7
AT4G26790.2	GDSL-like Lipase/Acylhydrolase superfamily protein
AT5G41040.1	HXXXD-type acyl-transferase family protein
AT5G19730.1	Pectin lyase-like superfamily protein
AT2G48130.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT2G23540.1	GDSL-like Lipase/Acylhydrolase superfamily protein
Sb02g001160	similar to Putative uncharacterized protein
Sb08g005415	Predicted protein
AT3G22600.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT2G19570.1	cytidine deaminase 1
AT2G34340.1	Protein of unknown function, DUF584
AT5G13930.1	Chalcone and stilbene synthase family protein
AT1G75050.1	Pathogenesis-related thaumatin superfamily protein
AT3G11430.1	glycerol-3-phosphate acyltransferase 5
AT3G16520.3	UDP-glucosyl transferase 88A1

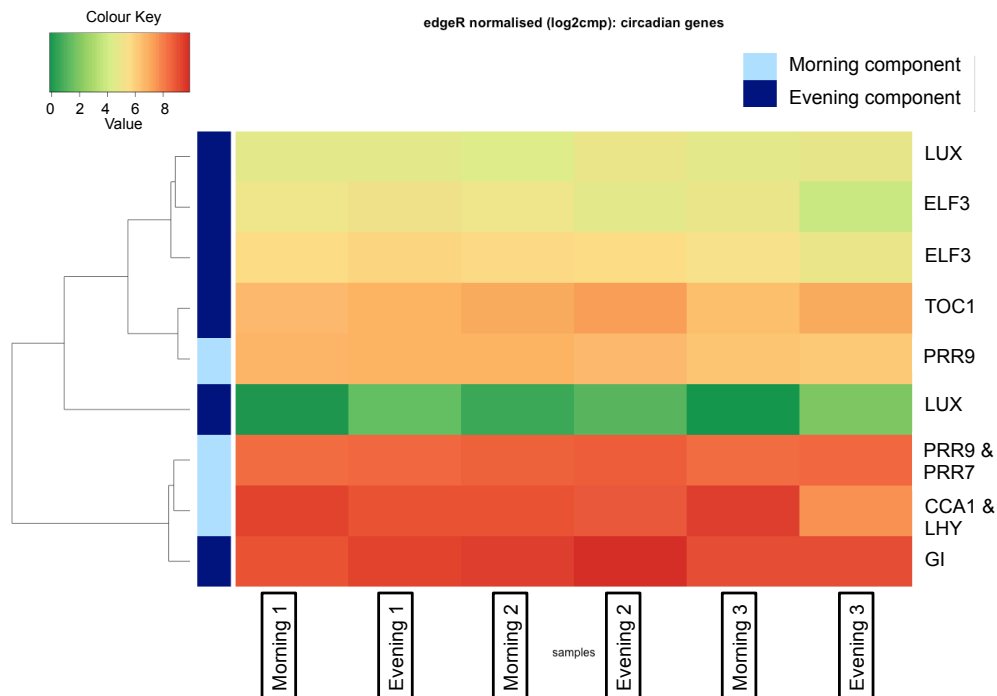
Sb09g030610	similar to Putative uncharacterized protein B1097D05.32
AT2G38110.1	glycerol-3-phosphate acyltransferase 6
AT1G53270.1	ABC-2 type transporter family protein
AT3G09220.1	laccase 7
AT3G22600.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT3G22600.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT5G61890.1	Integrase-type DNA-binding superfamily protein
Sb03g007770	similar to Putative uncharacterized protein
AT3G61460.1	brassinosteroid-responsive RING-H2
Sb06g031170	similar to OSIGBa0147J19.8 protein
AT5G24000.1	Protein of unknown function (DUF819)
AT3G25030.2	RING/U-box superfamily protein
AT4G13830.2	DNAJ-like 20
AT1G33260.2	Protein kinase superfamily protein
AT2G39980.1	HXXXD-type acyl-transferase family protein
AT3G07390.1	auxin-responsive family protein
AT3G05200.1	RING/U-box superfamily protein
AT4G01575.1	serine protease inhibitor, Kazal-type family protein
AT1G80290.2	Nucleotide-diphospho-sugar transferases superfamily protein
Sb03g032365	similar to Putative uncharacterized protein P0684B02.17
AT2G15760.1	Protein of unknown function (DUF1645)
AT4G15480.1	UDP-Glycosyltransferase superfamily protein
AT3G46090.1	C2H2 and C2HC zinc fingers superfamily protein
AT4G34380.1	Transducin/WD40 repeat-like superfamily protein
AT4G25470.1	C-repeat/DRE binding factor 2
AT5G23950.1	Calcium-dependent lipid-binding (CaLB domain) family protein
Sb04g030060	similar to Putative uncharacterized protein
AT1G52340.1	NAD(P)-binding Rossmann-fold superfamily protein
AT1G51760.1	peptidase M20/M25/M40 family protein
AT3G13620.1	Amino acid permease family protein
Sb04g034090	weakly similar to Putative uncharacterized protein
AT5G22090.2	Protein of unknown function (DUF3049)
AT5G01380.1	Homeodomain-like superfamily protein
Sb04g028310	similar to Os02g0741700 protein
AT4G11070.1	WRKY family transcription factor
Sb01g044630	similar to Os03g0188500 protein
AT2G22880.1	VQ motif-containing protein
AT3G54040.1	PAR1 protein



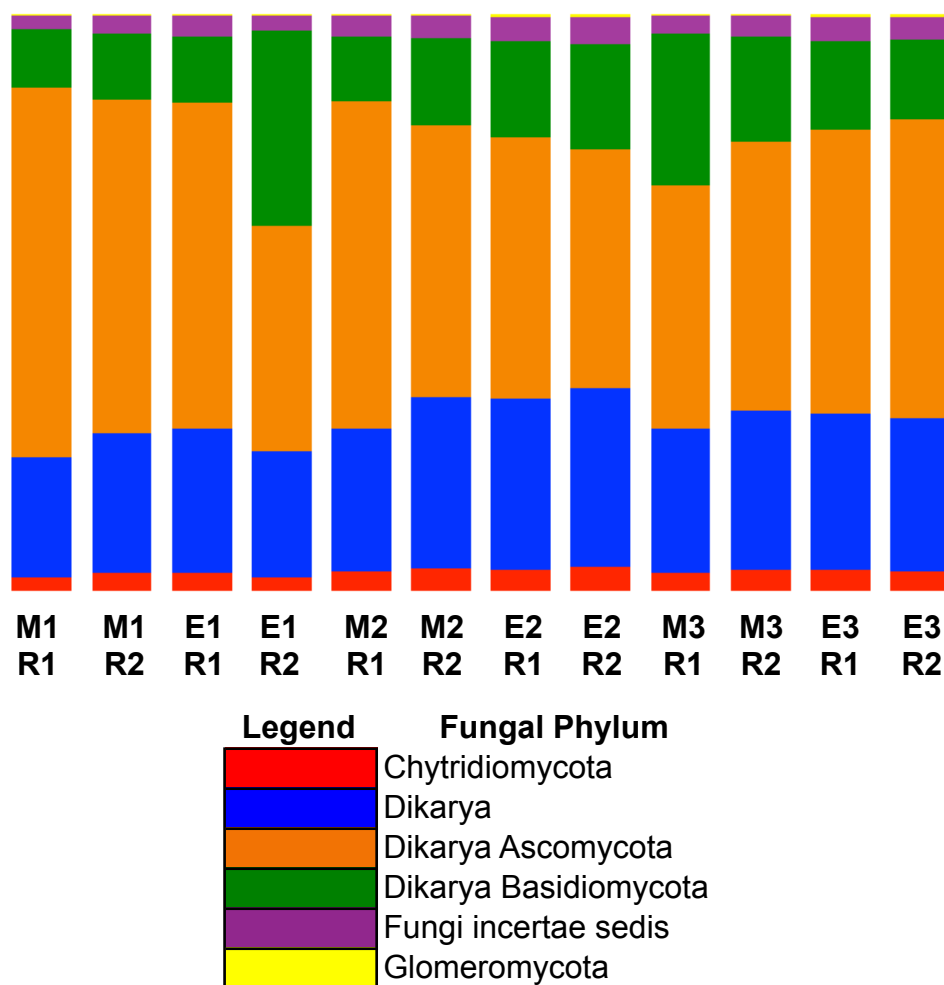




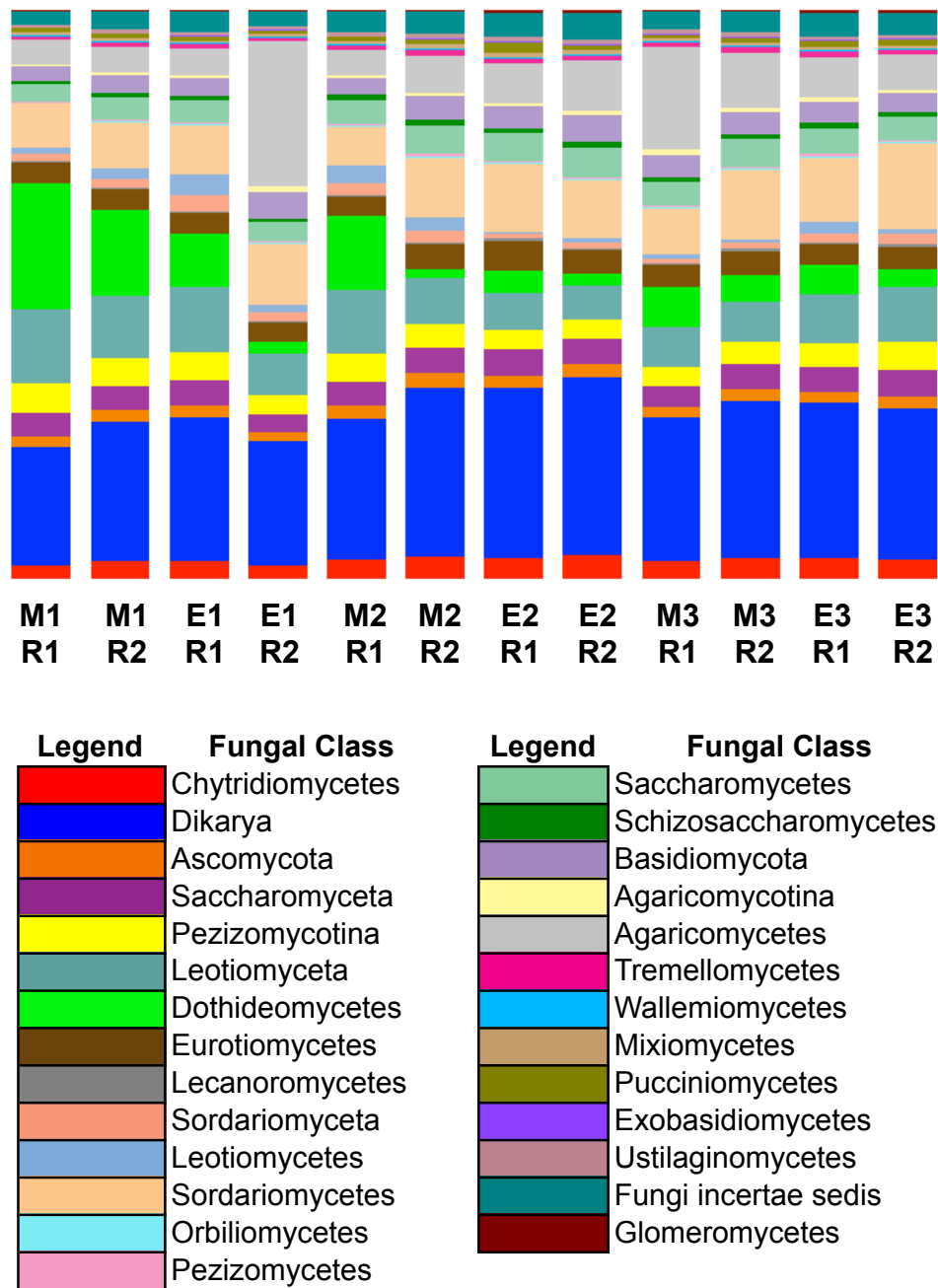
Appendix Figure 5.4 a - e Scaled gene expression heatmaps, for each of the five clusters: **a)** cluster 5 & 6, **b)** cluster 1, **c)** cluster 2, **d)** cluster 3, and **e)** cluster 4. Heatmaps are of DEGs using Z-values, over three consecutive days, replicates of morning and evening are merged.



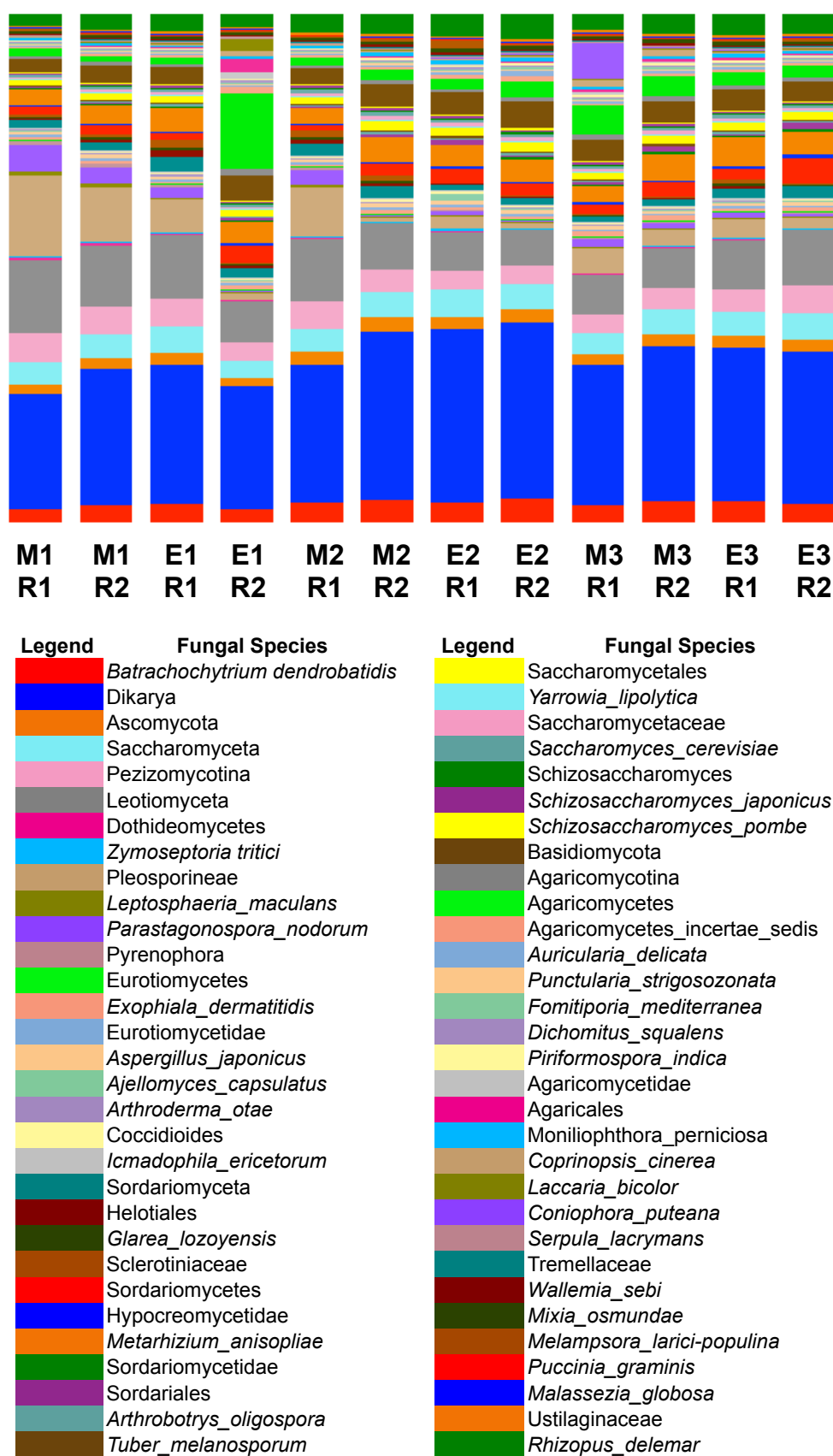
Appendix Figure 5.5 Heat map of circadian clock homologues gene expression, in the *M. giganteus* transcriptome, over three consecutive days. Replicates merged from each morning and evening. Expression profiles were ordered by hierarchical clustering. For gene identities see results section **Circadian clock gene homologues**.



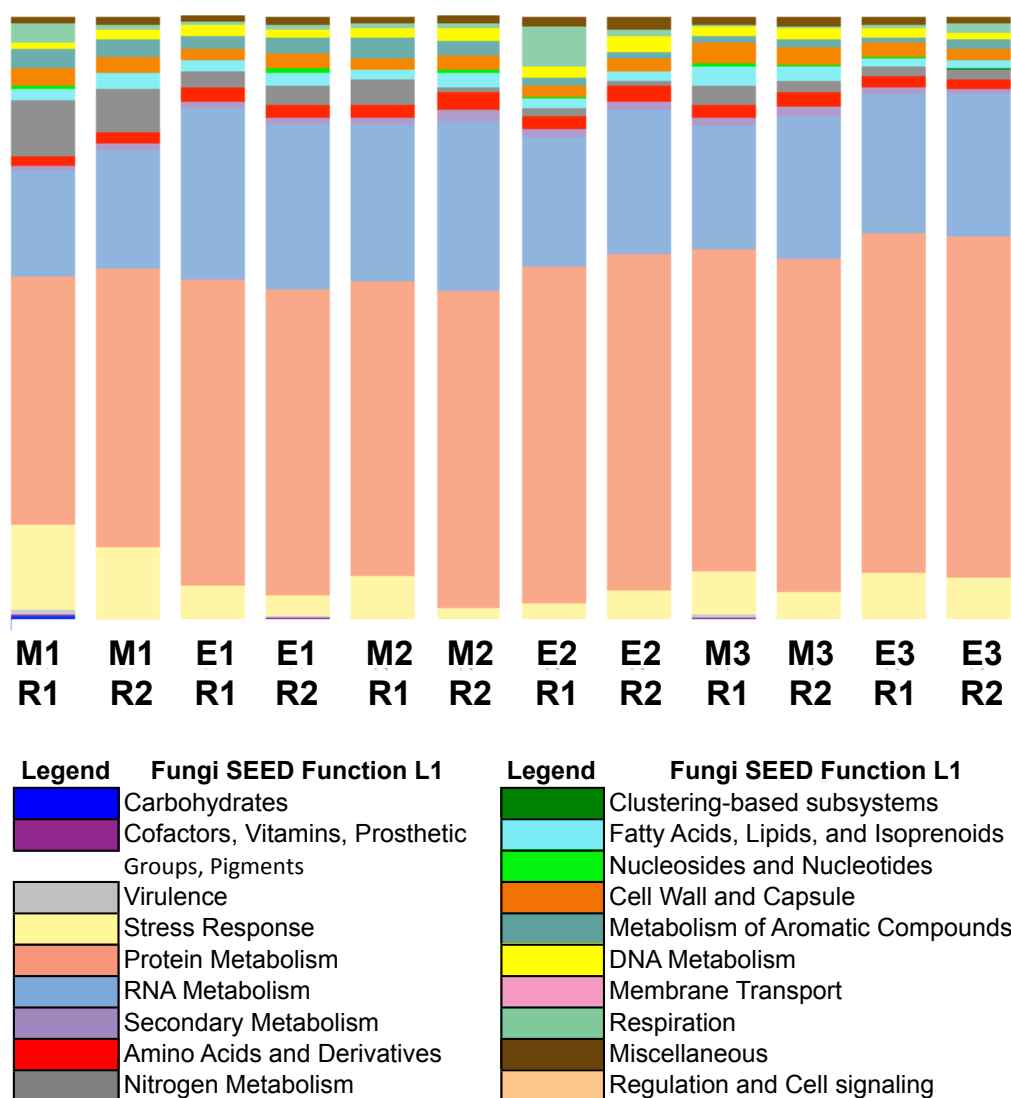
Appendix Figure 5.6a. Subsampled read proportions of fungal phyla, from the meta-transcriptome, in the morning (M) and evening (E) samples, showing both replicates (R1 and R2). The Dikarya group, represents reads conserved in Ascomycota and Basidiomycota, as reads were annotated using lowest common ancestor.



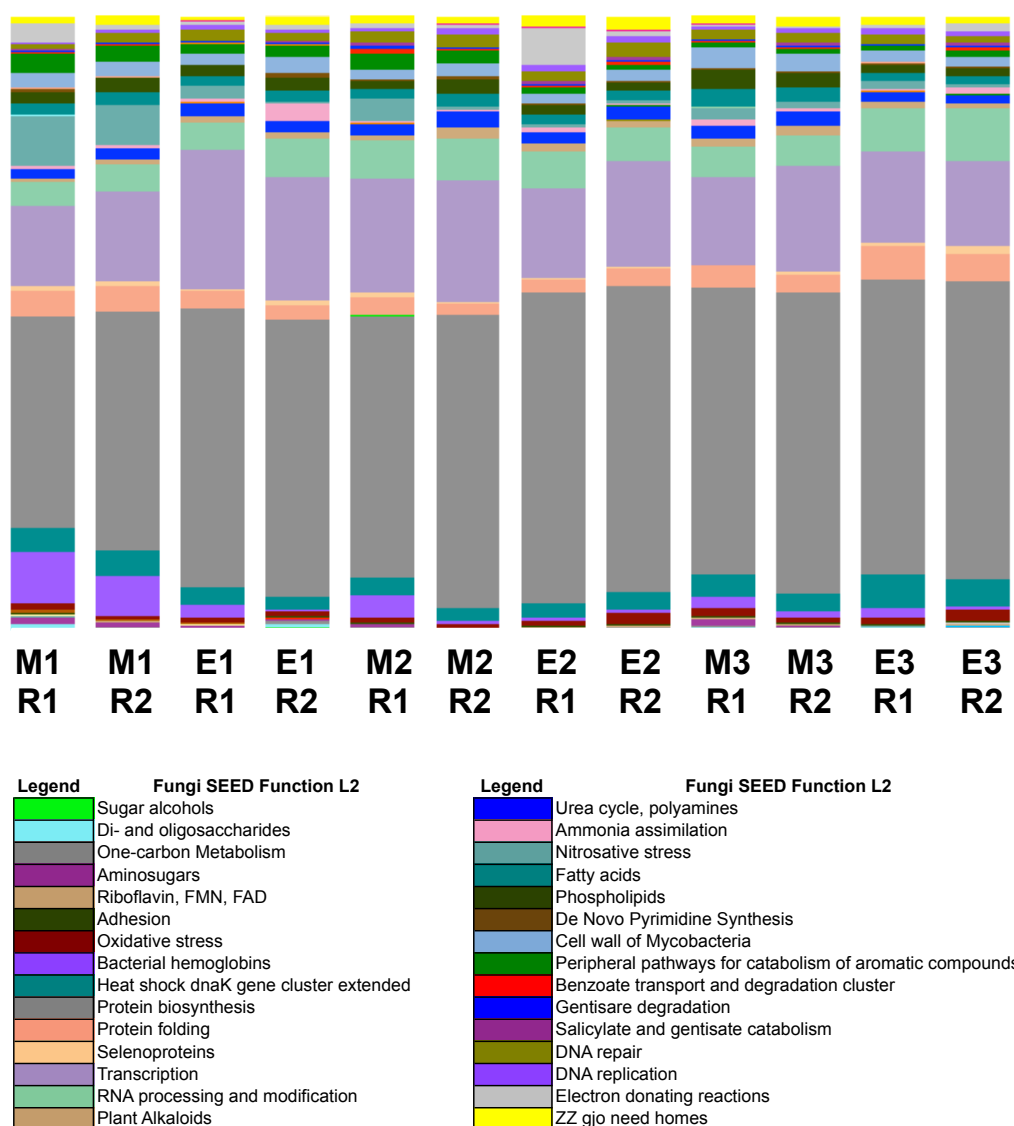
Appendix Figure 5.6b. Subsampled read proportions of fungal class, from the meta-transcriptome, in the morning (M) and evening (E) samples, showing both replicates (R1 and R2).



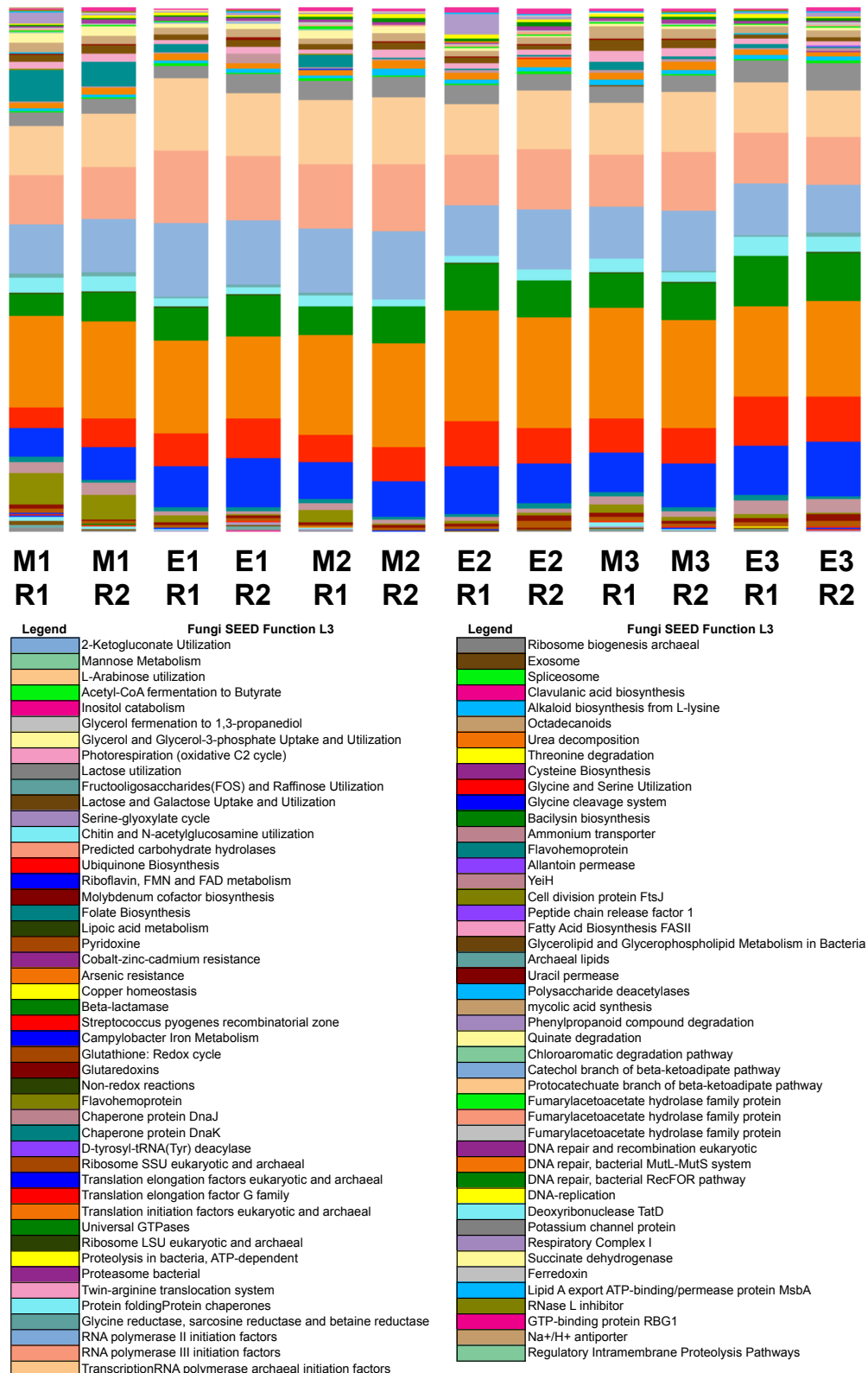
Appendix Figure 5.6c Subsampled read proportions of fungal species, from the meta-transcriptome, in the morning (M) and evening (E) samples, showing both replicates (R1 and R2).



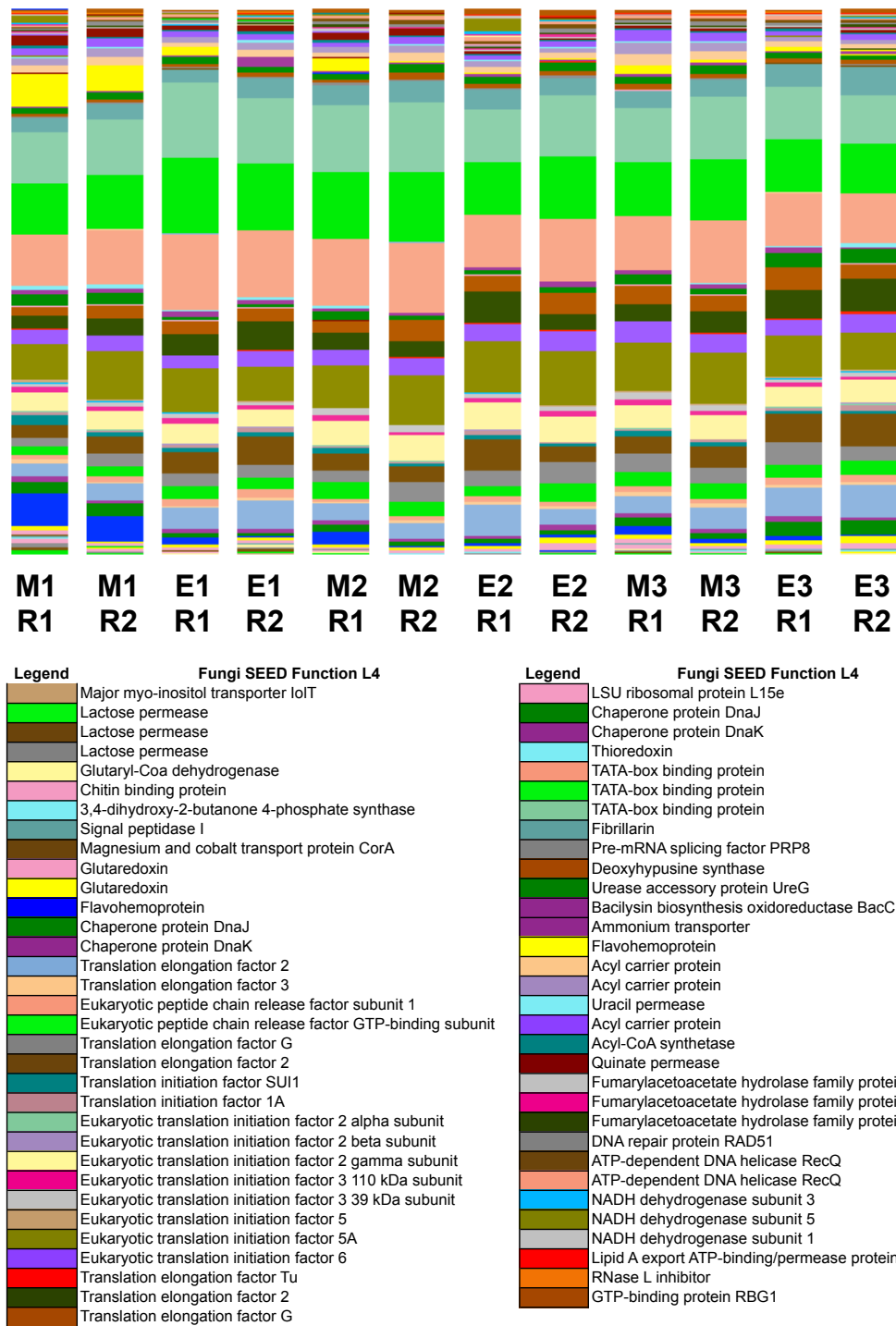
Appendix Figure 5.7a Subsampled Read proportions of SEED Level 1 annotation of morning (M) and evening (E), fungal functional gene expression, showing both replicates (R1 and R2).



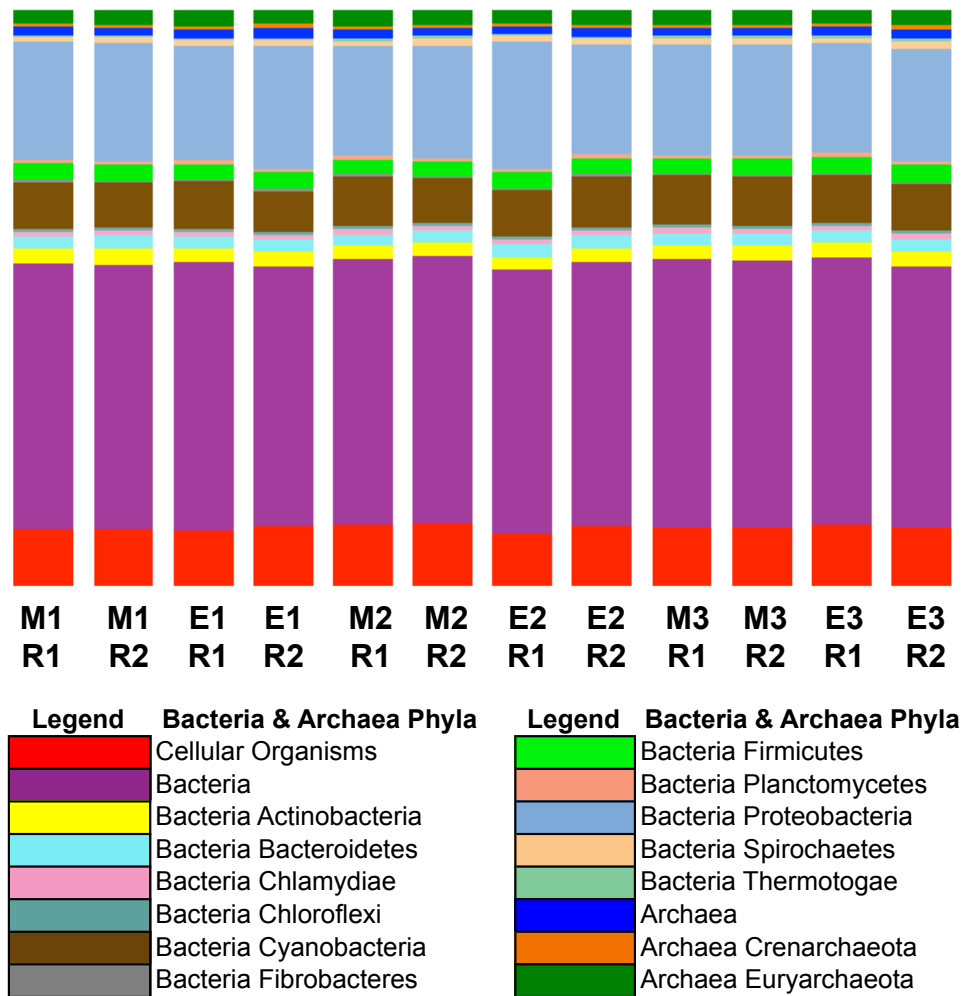
Appendix Figure 5.7b Subsampled read proportions of SEED Level 2 annotation of morning (M) and evening (E), fungal functional gene expression, showing both replicates (R1 and R2).



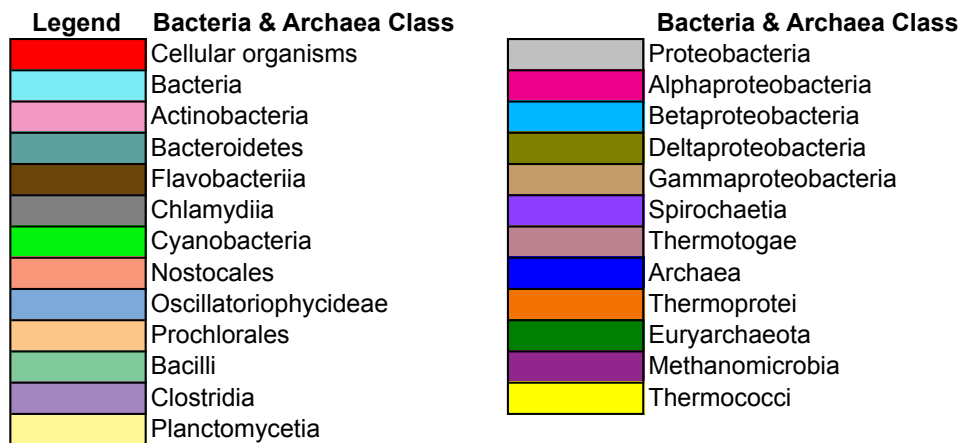
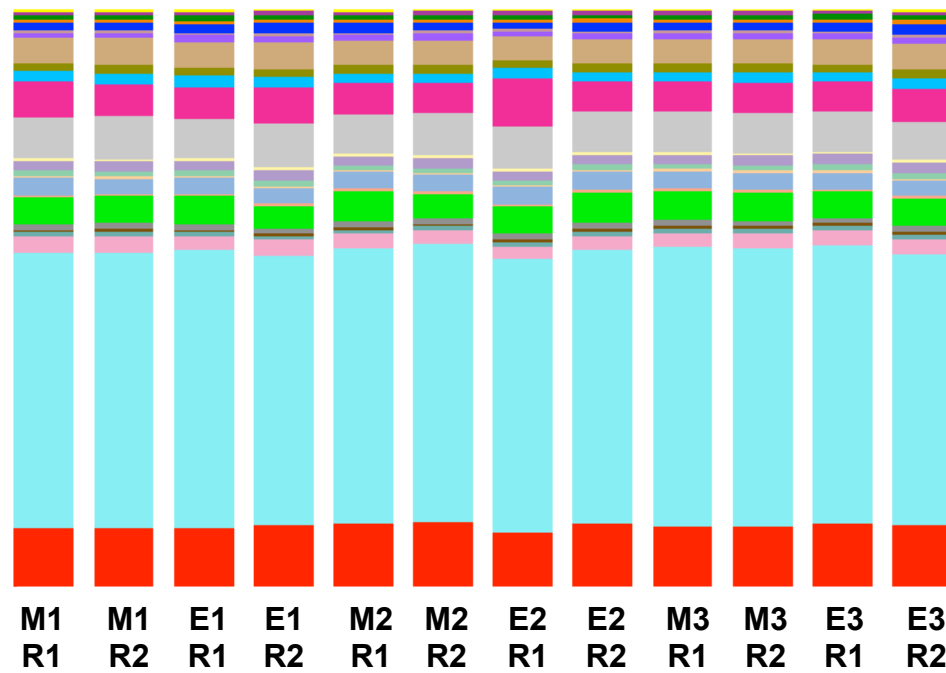
Appendix Figure 5.7c Subsampled read proportions of SEED Level 3 annotation of morning (M) and evening (E), fungal functional gene expression, showing both replicates (R1 and R2).



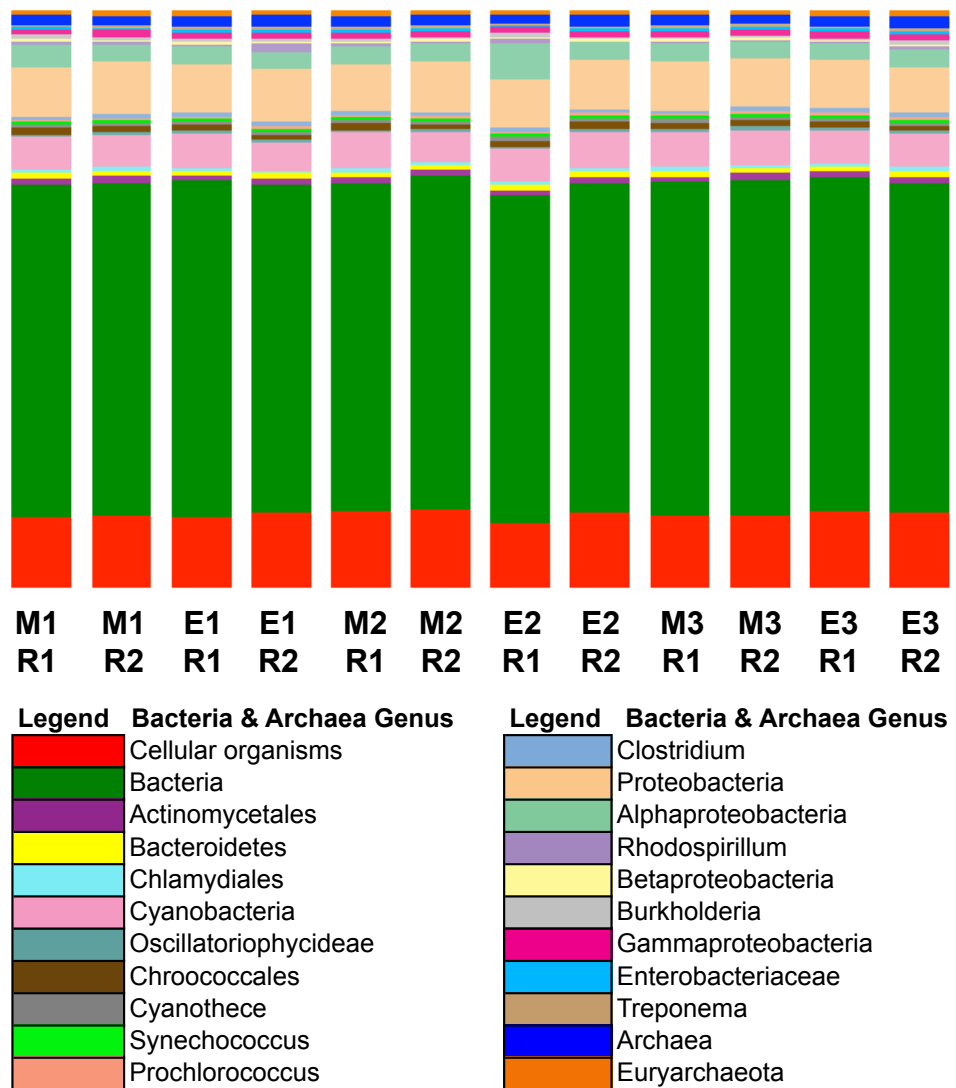
Appendix Figure 5.7d Subsampled read proportions of SEED Level 4 annotation of morning (M) and evening (E), fungal functional gene expression, showing both replicates (R1 and R2).



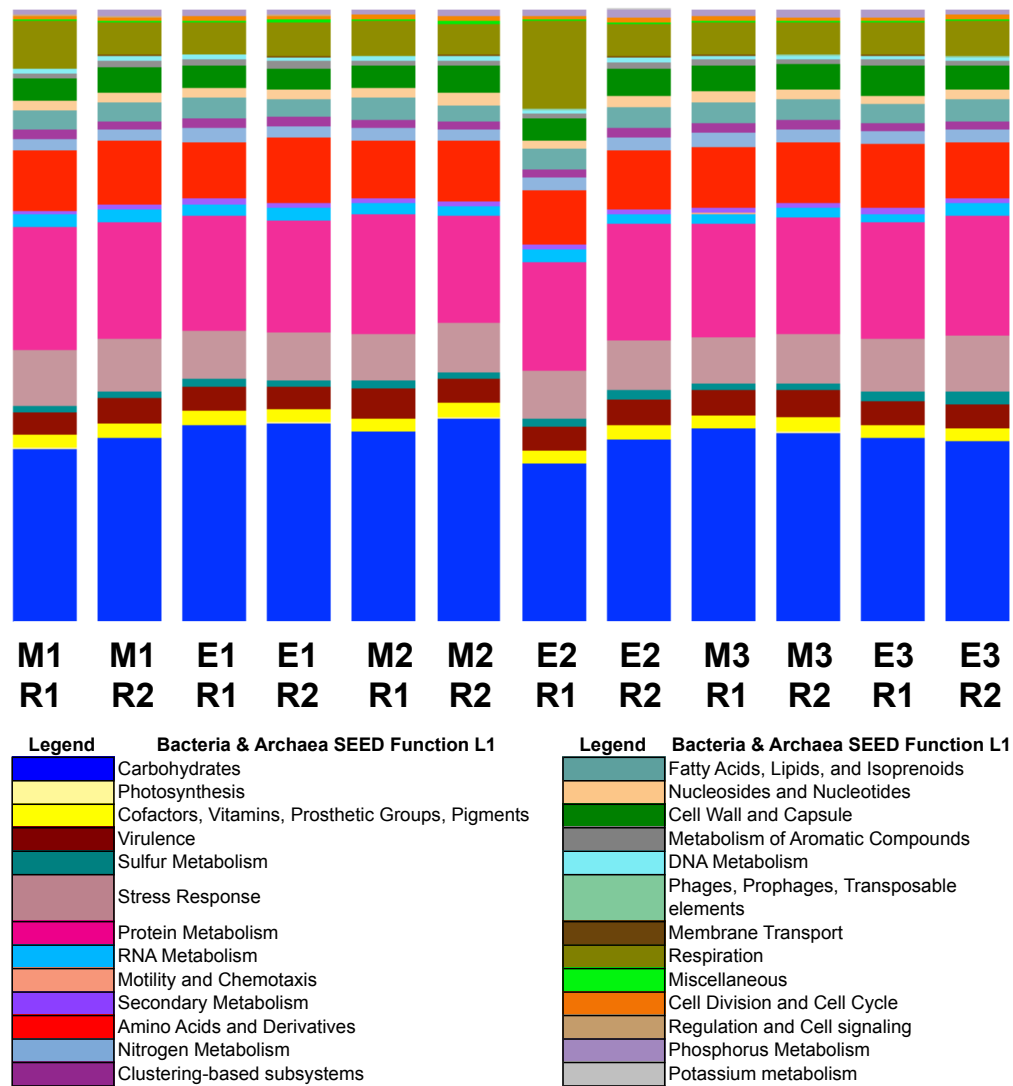
Appendix Figure 5.8a Subsampled read proportions of bacterial and archaeal taxonomy, phyla, from the meta-transcriptome, in the morning (M) and evening (E) samples, showing both replicates (R1 and R2).



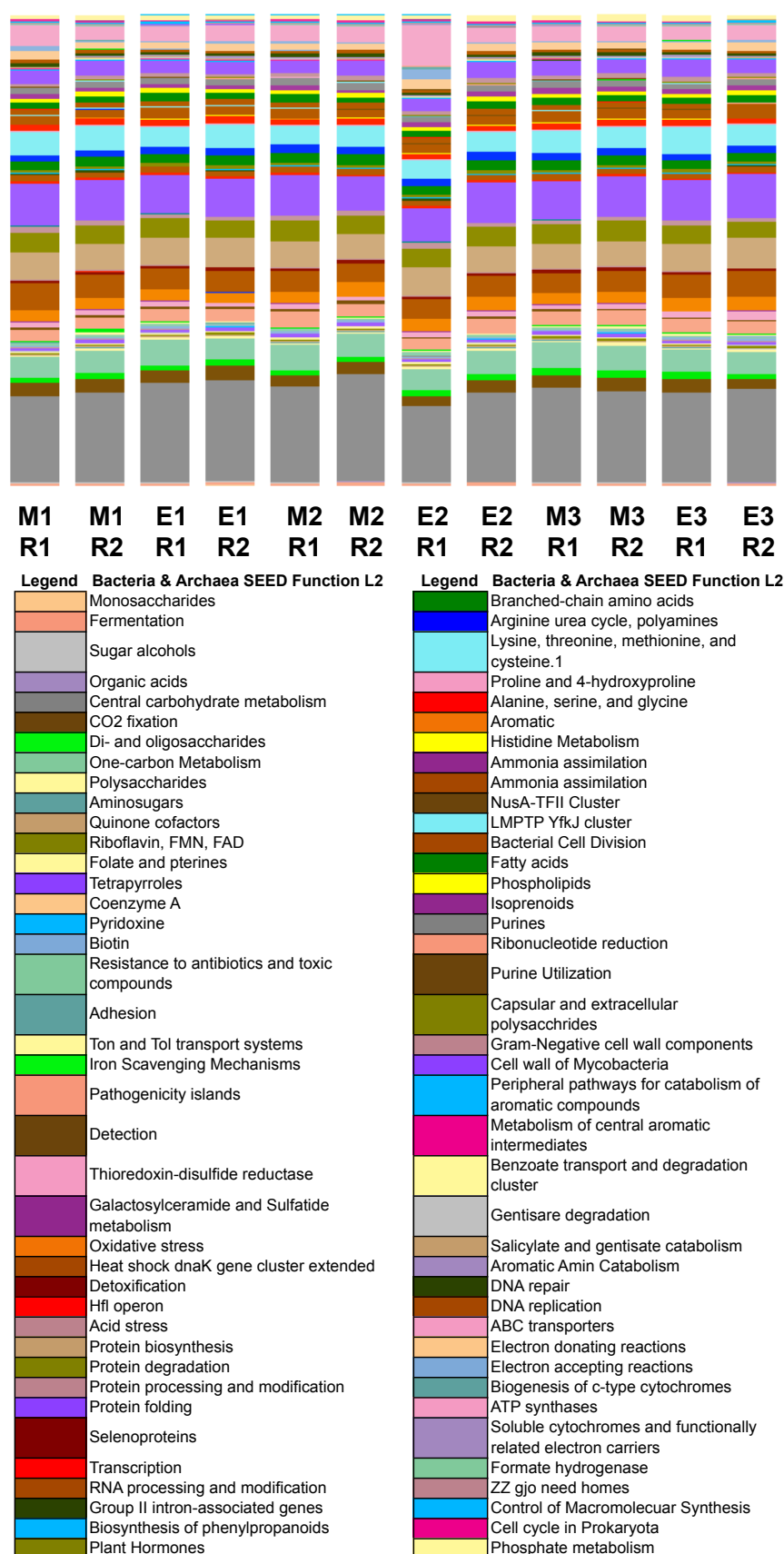
Appendix Figure 5.8b Subsampled read proportions of bacterial and archaeal taxonomy, class, from the meta-transcriptome, in the morning (M) and evening (E) samples, showing both replicates (R1 and R2).



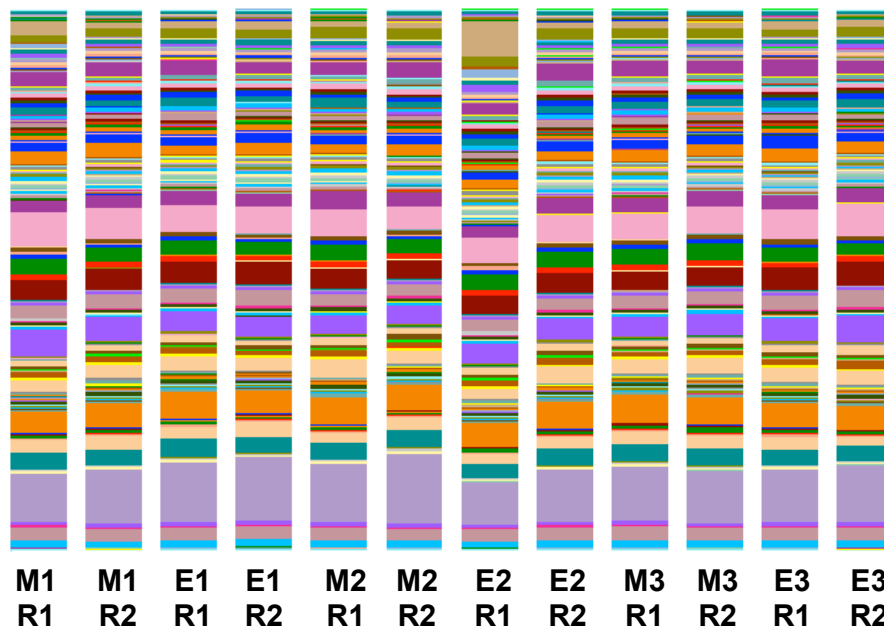
Appendix Figure 5.8c Subsampled read proportions of bacterial and archaeal taxonomy, genera, from the meta-transcriptome, in the morning (M) and evening (E) samples, showing both replicates (R1 and R2).



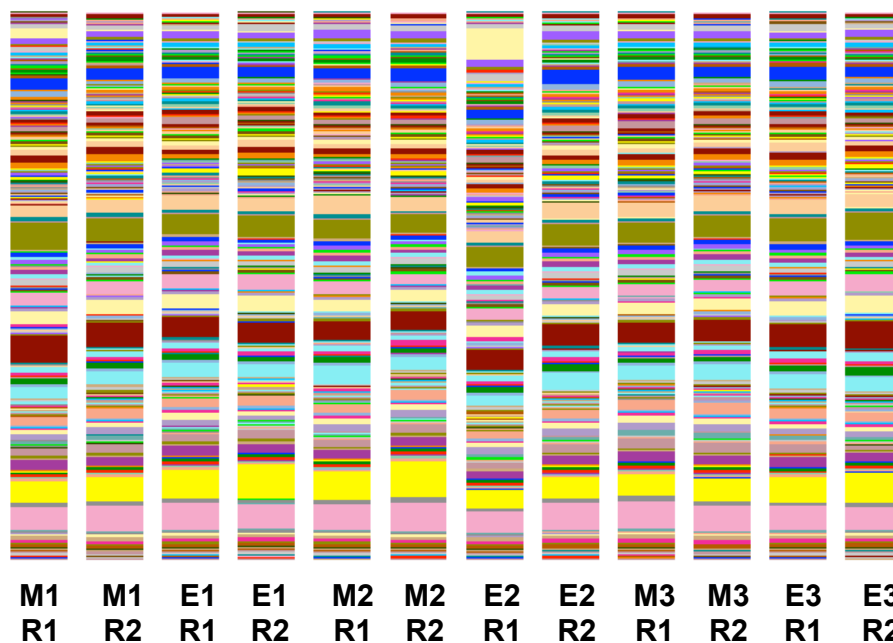
Appendix Figure 5.9a SEED Level 1 annotation of morning (M) and evening (E), bacterial and archaeal functional gene expression, showing both replicates (R1 and R2).



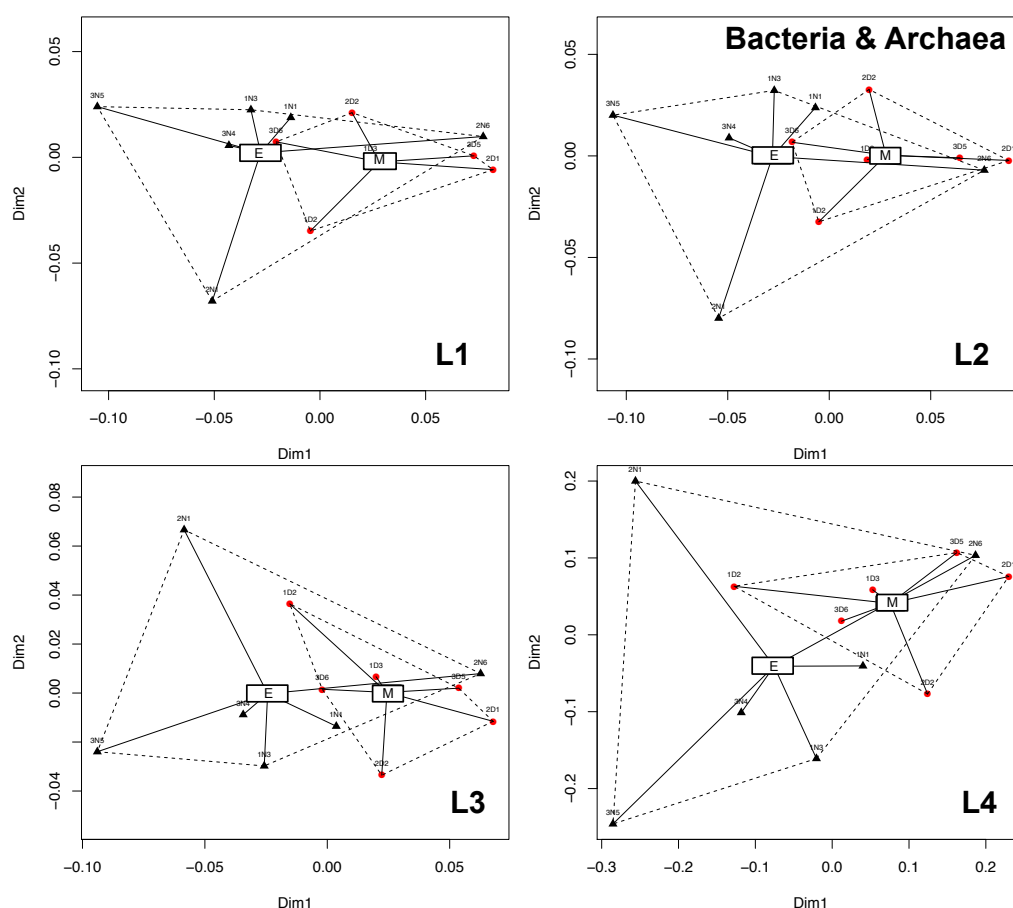
Appendix Figure 5.9b SEED Level 2 annotation of morning (M) and evening (E), bacterial and archaeal functional gene expression, showing both replicates (R1 and R2).



Appendix Figure 5.9c SEED Level 3 annotation of morning (M) and evening (E), bacterial and archaeal functional gene expression, showing both replicates (R1 and R2). There were 599 functions at L3 (legend too large to fit on page).



Appendix Figure 5.9d SEED Level 4 annotation of morning (M) and evening (E), bacterial and archaeal functional gene expression, showing both replicates (R1 and R2). There were 1550 functions at L4 (legend too large to fit on page).



Appendix Figure 5.10 NMDS plot of fungal morning (M) and evening (E) expressed genes, with SEED functional annotation, Level (L) 1 – 4, see Appendix Figure 5.9a–d for gene identities and abundances. Points represent community profiles of each sample, where red circles are morning samples, and black triangles are evening samples. In data point labels, the first number represents the sampling day (1-3), and the final number is the replicate number.

Appendix Table 5.2 ADONIS (Bray Curtis) differences between morning and evening fungal functions, separated into SEED Level (L) 1 – 4.

SEED Level	ADONIS (Bray Curtis)	
	r^2	P
L1	0.193	0.103
L2	0.166	0.105
L3	0.147	0.110
L4	0.134	0.109